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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
A61K 38/17, C07K 14/47, 16/18, C12N 5/02, 5/06, 15/12, 15/70, 15/79, C12Q 1/68, G01N 33/53

A1

(11) International Publication Number: **WO 96/26736**

(43) International Publication Date: 6 September 1996 (06.09.96)

(21) International Application Number: PCT/US96/02957

(22) International Filing Date: 1 March 1996 (01.03.96)

(30) Priority Data:

08/397,651	1 March 1995 (01.03.95)	US
08/469,427	6 June 1995 (06.06.95)	US
08/569,063	6 December 1995 (06.12.95)	US

(71) Applicants: LUDWIG INSTITUTE FOR CANCER RESEARCH [US/US]; 1345 Avenue of the Americas, New York, NY 10105 (US). HELSINKI UNIVERSITY LICENSING LTD., OY [FI/FI]; P.O. Box 26, FIN-00014 Helsinki (FI).

(72) Inventors: ERIKSSON, Ulf; Haegervaegen 27, S-746 34 Balsta (SE). OLOFSSON, Birgitta; Kling, Hornsgatan 106, 3 tr, S-117 26 Stockholm (SE). ALITALO, Kari; Nyyrikintie 4 A, FIN-02100 Espoo (FI). PAJUSOLA, Katri; Kasteholmantie 4 A 8, FIN-00900 Helsinki (FI).

(74) Agent: EVANS, Joseph, D.; Evenson, McKeown, Edwards & Lenahan, Suite 700, 1200 G Street N.W., Washington, DC 20005 (US).

(81) Designated States: AU, CA, CN, FI, JP, NO, NZ, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
With amended claims.

(54) Title: VASCULAR ENDOTHELIAL GROWTH FACTOR-B

(57) Abstract

VEGF-B polypeptides from the PDGF family of growth factors having the property of promoting mitosis and proliferation of vascular endothelial cells, DNA sequences encoding these polypeptides, pharmaceutical compositions containing them and antibodies which react with them. The VEGF-B polypeptides are useful in stimulating angiogenesis as well as in diagnostic applications.

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VASCULAR ENDOTHELIAL GROWTH FACTOR-B

Background of the Invention

Angiogenesis, or the proliferation of new capillaries from pre-existing blood vessels, is a fundamental process necessary for normal growth and development of tissues. It is a prerequisite for the development and differentiation of the vascular tree, as well as for a wide variety of fundamental physiological processes including embryogenesis, somatic growth, tissue and organ repair and regeneration, cyclical growth of the corpus luteum and endometrium, and development and differentiation of the nervous system. In the female reproductive system, angiogenesis occurs in the follicle during its development, in the corpus luteum following ovulation and in the placenta to establish and maintain pregnancy. Angiogenesis additionally occurs as part of the body's repair processes, e.g. in the healing of wounds and fractures. Angiogenesis is also a factor in tumor growth, since a tumor must continuously stimulate growth of new capillary blood vessels in order to grow.

Capillary blood vessels consist of endothelial cells and pericytes. These two cell types carry all of the genetic information to form tubes, branches and entire capillary networks. Specific angiogenic molecules can initiate this process. In view of the physiological importance of angiogenesis, much effort has been devoted to the isolation, characterization and purification of factors

characterized as to their molecular, biochemical and biological properties. For reviews of such angiogenesis regulators, see Klagsbrun et al., "Regulators of Angiogenesis", *Ann. Rev. Physiol.*, 53:217-39 (1991); and Folkman et al., "Angiogenesis," *J. Biol. Chem.*, 267:10931-934 (1992). Recent results have implicated several endothelial receptor tyrosine kinases (RTKs) in the establishment and maintenance of the vascular system.

One such growth factor, which is highly specific as a mitogen for vascular endothelial cells, is termed vascular endothelial growth factor (VEGF). See Ferrara et al., "The Vascular Endothelial Growth Factor Family of Polypeptides," *J. Cellular Biochem.*, 47:211 218 (1991); Connolly, "Vascular Permeability Factor: A Unique Regulator of Blood Vessel Function," *J. Cellular Biochem.*, 47:219-223 (1991). VEGF is a potent vasoactive protein that has been detected in media conditioned by a number of cell lines including bovine pituitary follicular cells. VEGF is a glycosylated cationic 46-48 kD dimer made up of two 24 kD subunits. It is inactivated by sulfhydryl reducing agents, resistant to acidic pH and to heating, and binds to immobilized heparin. VEGF is sometimes referred to as vascular permeability factor (VPF) because it increases fluid leakage from blood vessels following intradermal injection. It also has been called by the name vasculotropin.

Four different molecular species of VEGF have been detected. The 165 amino acid species has a molecular weight of approximately 46 kD and is the predominant molecular form found in normal cells and tissues. A less abundant, shorter form with a deletion of 44 amino acids between positions 116 and 159 ($VEGF_{121}$), a longer form with an insertion of 24 highly basic residues in position 116 ($VEGF_{189}$), and another longer form with an insertion of 41 amino acids ($VEGF_{206}$), which includes the 24 amino acid insertion found in $VEGF_{189}$,

of the isoforms of VEGF are biologically active. For example, each of the species when applied intradermally is able to induce extravasation of Evans blue.

The various species of VEGF are encoded by the same gene and arise from alternative splicing of messenger RNA. This conclusion is supported by Southern blot analysis of human genomic DNA, which shows that the restriction pattern is identical using either a probe for $VEGF_{165}$ or one which contains the insertion in $VEGF_{206}$. Analysis of genomic clones in the area of putative mRNA splicing also shows an intron/exon structure consistent with alternative splicing.

The different isoforms of VEGF have different chemical properties which may regulate cellular release, compartmentalization, bioavailability and possibly also modulate the signalling properties of the growth factors.

Analysis of the nucleotide sequence of the VEGF gene indicates that VEGF is a member of the platelet-derived growth factor (PDGF) family. VEGF and PlGF are ligands for two endothelial RTKs, flt-1 (VEGF receptor 1, VEGFR1) and flk-1/KDR (VEGF receptor 2, VEGFR2). The amino acid sequence of VEGF exhibits approximately 20% homology to the sequences of the A and B chains of PDGF, as well as complete conservation of the eight cysteine residues found in both mature PDGF chains. $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$ also contain eight additional cysteine residues within the carboxy-terminal region. The amino-terminal sequence of VEGF is preceded by 26 amino acids corresponding to a typical signal sequence. The mature protein is generated directly following signal sequence cleavage without any intervening prosequence. The existence of a potential glycosylation site at Asn^{74} is consistent with other evidence that VEGF is a glycoprotein, but the polypeptide has been reported to exist in both glycosylated and deglycosylated species.

Like other cytokines, VEGF can have diverse effects that depend on the specific biological context in which it

KDR/flk-1 are required for the formation and maintenance of the vascular system as well as for both physiological and pathological angiogenesis. VEGF is a potent endothelial cell mitogen and directly contributes to induction of angiogenesis *in vivo* by promoting endothelial cell growth during normal embryonic development, wound healing, and tissue regeneration and reorganization. VEGF is also involved in pathological processes such as growth and metastasis of solid tumors and ischemia-induced retinal disorders. A most striking property of VEGF is its specificity. It is mitogenic *in vitro* at 1 ng/ml for capillary and human umbilical vein endothelial cells, but not for adrenal cortex cells, corneal or lens epithelial cells, vascular smooth muscle cells, corneal endothelial cells, granulosa cells, keratinocytes, BHK-21 fibroblasts, 3T3 cells, rat embryo fibroblasts, human placental fibroblasts and human sarcoma cells. The target cell specificity of VEGF is thus restricted to vascular endothelial cells. VEGF can trigger the entire sequence of events leading to angiogenesis and stimulates angiogenesis *in vivo* in the cornea and in a healing bone graft model. It is able to stimulate the proliferation of endothelial cells isolated from both small and large vessels. Expression of VEGF mRNA is temporally and spatially related to the physiological proliferation of capillary blood vessels in the ovarian corpus luteum or in the developing brain. VEGF expression is triggered by hypoxia so that endothelial cell proliferation and angiogenesis appear to be especially stimulated in ischemic areas. VEGF is also a potent chemoattractant for monocytes. In addition, VEGF induces plasminogen activator and plasminogen activator inhibitor in endothelial cells.

Tumor cells release angiogenic molecules such as VEGF, and monoclonal antibodies to VEGF have been shown to inhibit the growth of certain types of tumor such as

Endothelial Growth Factor-Induced Angiogenesis Suppresses Tumor Growth *in vivo*," *Nature*, 362:841-844 (1993). This suggests that blocking VEGF action is of potential therapeutic significance in treating tumors in general, and highly-vascularized, aggressive tumors in particular.

Summary of the Invention

It is an object of the invention to provide a new growth factor having the property of promoting proliferation of endothelial cells.

Another object of the invention is to provide isolated DNA sequences which encode a new growth factor which promotes proliferation of endothelial cells.

It is also an object of the invention to provide new products which may be useful in diagnostic and/or therapeutic applications.

These and other objects are achieved in accordance with the present invention by providing an isolated DNA which codes for a protein exhibiting the following characteristic amino acid sequence (SEQ ID NO:16):

Pro-Xaa-Cys-Val-Xaa-Xaa-Xaa-Arg-Cys-Xaa-Gly-Cys-Cys

and having the property of promoting proliferation of endothelial cells or mesodermal cells, the DNA being selected from the group consisting of the DNA of Figures 1 and 2 (SEQ ID NO:1), the DNA of Figure 3 (SEQ ID NO:4), the DNA of Figure 5 (SEQ ID NO:6); the DNA of Figure 7 (SEQ ID NO:8), the DNA of Figure 10 (SEQ ID NO:10), the DNA of Figure 12 (SEQ ID NO:12), the DNA of Figure 14 (SEQ ID NO:14), and DNA's which hybridize under stringent conditions with at least one of the foregoing DNA sequences.

In accordance with further aspects of the invention, the objects are also achieved by providing a protein exhibiting the following characteristic amino acid sequence

Pro-Xaa-Cys-Val-Xaa-Xaa-Xaa-Arg-Cys-Xaa-Gly-Cys-Cys

(SEQ ID NO:16) and having the property of

which protein comprises a sequence of amino acids substantially corresponding to an amino acid sequence selected from the group consisting of the amino acid sequence of Figure 1 (SEQ ID NO:2), the amino acid sequence of Figure 2 (SEQ ID NO:3), the amino acid sequence of Figure 4 (SEQ ID NO:5), the amino acid sequence of Figure 6 (SEQ ID NO:7), the amino acid sequence of Figure 8 (SEQ ID NO:9), the amino acid sequenc of Figure 11 (SEQ ID NO:11), the amino acid sequence of Figure 13 (SEQ ID NO:13), and the amino acid sequence of Figure 15 (SEQ ID NO:15).

In further aspects of the invention, the objects are achieved by providing pharmaceutical preparations which comprise such proteins; and by providing antibodies which react with or recognize such proteins.

The novel growth factor of the present invention, referred to hereinafter as vascular endothelial growth factor B or VEGF-B, has close structural similarities to VEGF and to placenta growth factor (PlGF). All of the VEGF-B forms contain the characteristic amino acid sequence

Pro-Xaa-Cys-Val-Xaa-Xaa-Xaa-Arg-Cys-Xaa-Gly-Cys-Cys

(SEQ ID NO:16) (wherein Xaa represents a variable residue), which is an earmark of the PDGF/VEGF family of growth factors. This characteristic amino acid sequence can be found at amino acids 70 to 82 in Figures 4, 6, 8, 11, 13 and 15.

Clinical applications of the invention include diagnostic applications, acceleration of angiogenesis in wound healing, and inhibition of angiogenesis. Quantitation of VEGF-B in cancer biopsy specimens may be useful as an indicator of future metastatic risk. Topical application of VEGF-B preparations to chronic wounds may accelerate angiogenesis and wound healing. VEGF-B may be used in a manner analogous to VEGF.

According to yet further aspects of the invention, the objects are achieved by providing diagnostic/prognostic

one embodiment of the invention there is provided a diagnostic/prognostic test kit comprising antibodies to the new growth factor of the invention and means for detecting, and more preferably evaluating, binding between the antibodies and the new growth factor of the invention. In one preferred embodiment of the diagnostic/prognostic means according to the invention, either the antibody or the new growth factor is labelled, and either the antibody or the growth factor is substrate-bound, such that the growth factor-antibody interaction can be established by determining the amount of label attached to the substrate following binding between the antibody and the growth factor. In a particularly preferred embodiment of the invention, the diagnostic/prognostic means may be provided as a conventional ELISA kit.

In another alternative embodiment, the diagnostic/prognostic means may comprise PCR means for establishing the genomic sequence structure of a VEGF-B gene of a test individual and comparing this sequence structure with that disclosed in this application in order to detect any abnormalities, with a view to establishing whether any aberrations in VEGF-B expression are related to a given disease condition.

A yet further aspect of the invention concerns an antibody which recognizes VEGF-B and which is suitably labelled.

Another aspect of the invention concerns the provision of a pharmaceutical composition comprising either VEGF-B protein or antibodies thereto. Compositions which comprise VEGF-B protein may optionally further comprise either VEGF or heparin or both.

According to an additional aspect of the invention the manufacture of a medicament is provided which comprises VEGF-B protein and heparin for treating conditions characterized by lack of, or reduction in, angiogenesis

In another aspect, the invention relates to a protein dimer comprising VEGF-B protein, particularly a disulfide-linked dimer. The protein dimers of the invention include both homodimers of VEGF-B protein and heterodimers of VEGF-B and VEGF.

According to a yet further aspect of the invention there is provided a method for facilitating release of VEGF and/or VEGF-B from a cell comprising exposing a cell which expresses either or both of the aforementioned growth factors to heparin.

Another aspect of the invention involves providing a vector comprising an anti-sense nucleotide sequence which is complementary to at least a part of the DNA sequences disclosed herein which encode the new growth factor of the invention which promotes proliferation of endothelial cells. According to a yet further aspect of the invention such a vector comprising an anti-sense sequence may be used to inhibit, or at least mitigate, VEGF-B expression. The use of a vector of this type to inhibit VEGF-B expression is favored in instances where VEGF-B expression is associated with a disease such as in instances where tumors produce VEGF-B in order to provide for angiogenesis. Transformation of such tumor cells with a vector containing an anti-sense nucleotide sequence would suppress or retard angiogenesis and so would inhibit or retard growth of the tumor.

Brief Description of the Drawings

Figure 1 shows the nucleotide sequence of the (partial) cDNA clone of VEGF-B (SEQ ID NO:1) and the amino acid sequence of the protein segment (SEQ ID NO:2) coded by the first reading frame of the cDNA;

Figure 2 repeats the nucleotide sequence of the (partial) cDNA clone of VEGF-B (SEQ ID NO:1) and the amino acid sequence of the protein segment (SEQ ID NO:3) coded by the second reading frame of the cDNA;

Figure 3 shows the nucleotide sequence of the coding region of a full length cDNA clone of murine VEGF-B_{167} (SEQ ID NO:4);

Figure 4 shows the amino acid sequence of murine VEGF-B_{167} (SEQ ID NO:5);

Figure 5 shows the nucleotide sequence of the coding region of a cDNA clone of VEGF-B_{174} (SEQ ID NO:6);

Figure 6 shows the amino acid sequence of VEGF-B_{174} (SEQ ID NO:7);

Figure 7 shows the nucleotide sequence of a cDNA clone of VEGF-B_{112} (SEQ ID NO:8);

Figure 8 shows the amino acid sequence of VEGF-B_{112} (SEQ ID NO:9);

Figure 9 shows a comparison of the amino acid sequences of mVEGF-B_{167}, mVEGF$_{164}$, hPlGF, mPDGF A, and mPDGF B;

Figure 10 shows the nucleotide sequence of a clone of human VEGF-B_{167} (SEQ ID NO:10);

Figure 11 shows the amino acid sequence of human VEGF-B_{167} (SEQ ID NO:11); and

Figure 12 shows the nucleotide sequence of murine VEGF-B_{186} (SEQ ID NO:12);

Figure 13 shows the amino acid sequence of murine VEGF-B_{186} (SEQ ID NO:13);

Figure 14 shows the nucleotide sequence of human VEGF-B_{186} (SEQ ID NO:14);

Figure 15 shows the amino acid sequence of human VEGF-B_{186} (SEQ ID NO:15);

Figure 16 shows an amino acid sequence comparison of murine and human VEGF-B_{167} and VEGF-B_{186} isoforms (SEQ ID NOS: 5, 11, 13 & 15).

Figure 17 shows the schematic structure of mouse and human genes for VEGF-B;

Figure 18 shows a hydrophilicity analysis of murine VEGF-B_{167} and VEGF-B_{186} isoforms;

Figure 19 shows a phylogenetic analysis of the

Figure 20 is a graph showing the induction of [^{3}H]thymidine incorporation by VEGF-B, VEGF and bFGF for human umbilical vein endothelial cells (HUVEC) and bovine capillary endothelial (BCE) cells;

Figure 21 is a Northern blot analysis of the expression of VEGF-B_{186} transcripts in several mouse and human tissues;

Figure 22 shows the results of immunoprecipitation and SDS-PAGE analysis of cell culture media and detergent solubilized cell lysates from Cos-1 cells transiently transfected with a murine VEGF-B cDNA;

Figure 23A shows the results of immunoprecipitation and SDS-PAGE analysis of cell culture media from transfected Cos-1 cells separately expressing murine VEGF-B_{186} and human $VEGF_{165}$;

Figure 23B shows the results of immunoprecipitation and SDS-PAGE analysis of cell culture media (M) and detergent solubilized cell lysates (L) of Cos-1 cells which coexpress murine VEGF-B_{186} and human $VEGF_{165}$;

Figure 23C shows the results of immunoprecipitation and SDS-PAGE analysis of cell culture media from Cos-1 cells expressing murine VEGF-B_{186} and human VEGF, either separately or in combination, and from mock transfected control cells;

Figure 24 is a schematic illustration of the derivation of VEGF-B promoter-reporter clones; and

Figure 25 shows the nucleotide sequence of a 1.55 kb human VEGF-B promoter fragment (SEQ ID NO:17).

Detailed Description of Preferred Embodiments

The present invention thus is directed to new vascular endothelial growth factors, hereinafter referred to as VEGF-B growth factors, which share the angiogenic and other properties of VEGF, but which are distributed and expressed in tissues differently from VEGF.

VEGF-B growth factors are members of the family of platelet derived growth factors and are a growth factors

endothelial cells and/or mesodermal cells. They are produced by expression of DNA sequences which correspond to, or which are hybridizable under stringent conditions with, any one of the DNA sequences depicted in Figures 1 and 2 (SEQ ID NO:1), Figure 3 (SEQ ID NO:4), Figure 5 (SEQ ID NO:6), Figure 7 (SEQ ID NO:8), Figure 10 (SEQ ID NO:10), Figure 12 (SEQ ID NO:12) or Figure 14 (SEQ ID NO:14). It is intended to include within the scope of the invention all angiogenic proteins encoded by DNA sequences which hybridize under stringent conditions to any one of the foregoing DNA sequences. Suitable hybridization conditions include, for example, 50% formamide, 5 x SSPE buffer, 5 x Denhardts solution, 0.5% SDS and 100 μg/ml of salmon sperm DNA at 42°C overnight, followed by washing 2 x 30 minutes in 2 x SSC at 55°C.

The invention is also directed to an isolated and/or purified DNA which corresponds to, or which hybridizes under stringent conditions with, any one of the foregoing DNA sequences.

In a further aspect, the invention is directed to antibodies of VEGF-B growth factors, and particularly to monoclonal antibodies.

VEGF-B proteins are believed to interact with protein tyrosine kinase growth factor receptors. Details of such receptors are known in the art [See e.g. Wilks, A.F., "Protein Tyrosine Kinase Growth Factor Receptors and Their Ligands in Development, Differentiation, and Cancer," *Adv. Cancer Res.*, 60:43-73 (1993)].

Various adult mouse tissues were tested for expression of transcripts corresponding to VEGF-B by Northern blotting. The size of the mRNA was 1.3-1.4 kb. A mouse multiple tissue Northern blot (MTN, Clontech) was probed with the ~0.9 kb SalI/NotI fragment derived from the pPC67 yeast expression vectors described above. The probe was labelled with ^{32}P-dCTP using random priming (specific activity 10^{9}-10^{9}

using 50% formamide, 5 x SSPE buffer, 2% SDS, 10 x Denhardts solution, 100 μg/ml salmon sperm DNA and 1x10⁶ cpm of the labelled probe/ml. The blot was washed at room temperature for 2 x 30 min in 2 x SSC containing 0.05% SDS and then for 2 x 20 min at 52°C in 0.1 x SSC containing 0.1% SDS. The blot was then exposed at -70°C for three days using intensifying screens. Kodak XAR film was used. The relative expression levels as determined by visual examinations of the film are listed in the following table:

Table 1

Distribution of VEGF-B Transcripts in the Adult Mouse

Tissue	Relative Expression Level
Heart	+++++
Brain	+++
Spleen	(+)
Lung	++
Liver	+
Skeletal Muscle	++++
Kidney	+++
Testis	(+)

+++++ = very strong expression; ++ = rather weak expression;
++++ = strong expression; + = weak expression;
+++ = moderate expression; (+) = very weak expression.

A human multiple tissue Northern blot (MNT) from Clontech was probed using the murine partial cDNA to determine relative VEGF-B expression levels in various human tissues. The size of the transcript was 1.3-1.4 kb. The conditions were identical to those used for the mouse Northern blot described above. The relative VEGF-B transcript levels for the human Northern blot are listed in

also lists relative expression level data from the literature for VEGF in various mammalian systems.

Table 2

Tissues	Relative Expression Levels			
	VEGF-B (Northern blot)	VEGF (from literature)		
	human	human	murine	guinea pig
heart	+++++	++	+++	+++
brain	+		+	+
placenta	+			
lung	+	++++		++
liver	(+)	++	(+)	+
skeletal muscle	++++		+++	+
kidney	+	++	+	++
pancreas	+++			
spleen	++		-	+
thymus	+		-	
prostate	+++			
testis	++			(+)
ovary	+++			-
small intestine	++			
colon	+++			
peripheral blood leucocytes	+			

From a comparison of Table 1 and Table 2 it can be seen that mouse and human tissue expression levels of VEGF-B transcripts are relatively similar with the highest expression levels being found in heart and skeletal muscle. Significant differences may be seen in brain and kidney

large proportion of both muscular and epithelial cells, such as prostate, pancreas and colon from which some of the most common human tumors originate, express relatively high levels of VEGF-B.

A comparison of the relative expression levels of VEGF and VEGF-B in human tissues shows some striking differences. VEGF is expressed rather weakly by human heart tissue, but VEGF-B is very strongly expressed by the same tissue. On the other hand, VEGF is strongly expressed by human lung tissue, but VEGF-B is only weakly expressed by human lung tissue. In a similar vein, human liver tissue expresses VEGF at a moderate level, but VEGF-B is expressed only very weakly. These data evidence that despite their general similarities, the actions of VEGF and VEGF-B are not completely identical.

The expression of VEGF-B transcripts was further analyzed in mouse and human tissues by Northern blotting and compared with the expression of VEGF transcripts. Mouse and human multiple tissue Northern (MTN) blots (Clontech) were hybridized with a ^{32}P-labelled mouse VEGF-B probe (≈0.9 kb SalI/NotI insert of the clone pcif 2). VEGF expression was analyzed with ^{32}P-labelled $VEGF_{165}$ cDNA as the probe. The hybridizations were carried out at 42°C in 50% deionized formamide, 5 x SSC pH 7.0, 1% SDS, 5 x Denhardt's solution and 100 μg/ml of denatured salmon sperm DNA. The filters were washed 2 x 30 min at 52°C in 2 x SSC containing 0.5% SDS and exposed to Kodak XAR film for 2-5 days at -70°C using intensifying screens. In situ hybridization analysis of adult mouse tissues from CBA mice and of embryos derived from matings of CBA and NMRI mice were carried out essentially as previously described by Korhonen et al., *Blood*, 80, 2548-55 (1992). The RNA probes (a 383 bp antisense probe and a 169 bp sense probe) were generated from a linearized plasmid containing a 440 bp Sal I/Sac I fragment derived from the pcif 2 cDNA clone. Radiolabelled

[^{35}S]UTP (Amersham Inc.). Alkaline hydrolysis of the probes was omitted. Hematoxylin was used for counterstaining. Control hybridizations with sense strand and RNAse A-treated sections did not give signals above background.

In mouse tissues the most abundant expression of the 1.4 kb VEGF-B transcript was detected in heart, brain, skeletal muscle, and kidney. The major 3.7 kb VEGF transcript was is expressed in heart, brain, lung, skeletal muscle and kidney. In human tissues, the most abundant expression of the 1.4 kb VEGF-B transcript and the major 3.7 and 4.5 kb VEGF transcripts were detected in heart, skeletal muscle, pancreas and prostate. Thus, although clear quantitative differences exist, it appears that VEGF B and VEGF are coexpressed in many human and mouse tissues.

The expression of VEGF-B transcripts was further examined by in situ hybridization in sections from adult mouse heart and skeletal muscle and from the early (E 10) mouse embryo. In the adult heart, VEGF-B transcripts are prominently expressed in the myocardium, while no specific signal is detected in arterial smooth muscle. In adult striated muscle, VEGF-B transcripts are expressed by some of the myofibers whereas others seem to lack the transcript. In the E 10 mouse embryo, VEGF-B transcripts are detected mainly in the developing heart. The myocardium of the adult mouse heart has a prominent signal. In striated muscle, VEGF-B expression is seen in subpopulations of myofibers. Strong signals were also obtained in the developing heart of the E10 mouse embryo. Other embryonic structures expressed lower or undetectable levels of transcripts for VEGF-B. Taken together, these tests indicate that VEGF-B transcrips are expressed primarily in muscular tissues. VEGF-B is particularly abundant in heart and skeletal muscle and is co-expressed with VEGF in these and other tissues. In transfected cells, VEGF-B forms cell surface associated, disulfide-linked homodimers and heterodimers with VEGF [illegible]

VEGF-B_{186} transcripts in several mouse and human tissues using a VEGF-B_{166} isoform specific probe is shown in Figure 21.

The chromosomal location of the VEGF-B gene was assessed by Southern blotting and polymerase chain reaction analysis of somatic cell hybrids and flucrescense *in situ* hybridization (FISH) of metaphase chromosomes. The VEGF-B gene was found on chromosome 11q13, proximal of the cyclin D1 gene. It is interesting that although the cyclin D1 gene is amplified in a number of human carcinomas, the VEGF-B gene was not amplified in several mammary carcinoma cell lines which contained amplified cyclin D1. Nevertheless, mutations in the VEGF-B gene may be related to vascular malformations and/or cardiovascular diseases.

Unless otherwise indicated, the following Examples used standard molecular biology techniques and procedures as disclosed in Ausubel et al. (eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, New York (1992).

Example 1: Partial cDNA clone with two reading frames.

A partial cDNA clone encoding murine VEGF-B was identified as follows. A cDNA library (E 14.5) derived from poly A+ mRNA isolated from 14.5 day old mouse embryos [Chevray P. and Nathans D., "Protein interaction cloning in yeast: Identification of mammalian proteins that react with the leucine zipper of Jun," *Proc. Natl. Acad. Sci. USA*, 89:5789-93 (1992)] was screened for cellular proteins which potentially might interact with cellular retinoic acid-binding protein type 1 (CRABP-I) using a yeast two-hybrid interaction trap screening technique as described by Gyuris J., Golemis E., Chertkov H. and Brent R., "Cdi1, a Human G1 and S Phase Protein Phosphatase That Associates with Cdk2," *Cell*, 75:791-803 (1993). This screening technique involves a fusion protein that contains a binding domain and that is known to be transcriptionally inert (the "bait"); reporter genes that have no basal transcription and that are bound by

expressed as chimeras and whose amino termini contain an activation domain and other useful moieties (the "prey"). The screened library was a 14.5 day mouse embryo plasmid library in the yeast expression vector pPC67 obtained from Dr. Pierre Chevray of the Johns Hopkins University, School of Medicine, 725 North Wolfe St., Baltimore, MD 21205. A positive cDNA clone (pcif-2) was recovered from the screening. The positive clone was sequenced using well known, conventional techniques and found to encode a protein highly homologous to VEGF and the other members of the PDGF family of growth factors. The ≈0.9 kb SalI/NotI insert in the plasmid pPC67 was cloned into pBluescript and fully sequenced using T7 and T3 vector primers together with internal primers. The plasmid pBluescript is commercially available from Stratagene Inc., LaJolla, California. The cDNA insert was found to be 886 base pairs long and to encode two polypeptides in different reading frames which were homologous to the N-terminal end and the C-terminal end, respectively, of VEGF. This novel growth factor is referred to hereinafter as VEGF-B. The clone is partial and lacks several amino acids in the amino terminal region and the entire signal sequence.

Figure 1 shows the nucleotide sequence (SEQ ID NO:1) of this partial cDNA clone of VEGF-B and the amino acid sequence (SEQ ID NO:2) encoded in the first reading frame thereof. The DNA sequence of Figure 1 was obtained by conventional sequencing of a clone (pcif-2) in the yeast expression vector pPC67. The clone comprised 886 base pairs and encoded a part of murine VEGF-B.

The isolated cDNA sequence will hybridize with the mammalian genomic DNA, e.g. either murine or human, which contains the VEGF-B gene. In addition to the coding sequence, the genomic DNA will contain one or more promoter sequence(s) which give and direct expression of VEGF-B in one or more specific tissues. Thus the coding sequence of

which in turn are specific to certain types of muscle fibers.

The nucleotide sequence is translated in two different reading frames into two different amino acid sequences. There is a stop codon (TGA) within the coding sequence at base pairs 309-311. Thus, VEGF-B comes in several splicing variants. The 5'end of the cloned cDNA sequence encodes an 102 amino acid long protein with significant homology to the N-terminal domains of VEGF, PlGF and PDGF A and B. In particular, a number of cysteine residues are perfectly conserved within this group of proteins. In addition to the nucleotide sequence (SEQ ID NO:1), Figure 1 further depicts the deduced amino acid sequence (SEQ ID NO:2) of this first protein.

Translation of the C-terminal end of the cDNA (base pairs 308-475) in a different reading frame results in a protein which is highly homologous to the C-terminal part of $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$. Figure 2 again shows the nucleotide sequence (SEQ ID NO:1) of Figure 1, but this time includes the deduced amino acid sequece (SEQ ID NO:3) of the second protein, which is encoded in the second reading frame and is 54 amino acids long. It thus appears that the VEGF-B gene encodes different proteins using alternative splicing of the primary transcript. The last part of the clone, encoding the second peptide might be expressed as a functional protein in other spliced variants of VEGF-B.

The aforedescribed proteins may exist in combined association with an additional N-terminal sequence of approximately five (5) to ten (10) amino acids, as well as a further leader sequence of approximately twenty-one (21) to twenty-eight (28) amino acids. Inasmuch such combined amino acid sequences exhibit the property of promoting the proliferation of endothelial cells and the DNA sequences which code for such combined peptide sequences will hybridize under stringent conditions with the DNA sequence

which codes for them are expressly contemplated to be within the scope of the present invention.

Example 2: Cloning of full length cDNA's for mouse VEGF-B.

Using the approximately 0.9 kb SalI/NotI cDNA insert of the previously identified cDNA clone of Example 1 as a probe, an adult mouse heart lambda ZAP-II cDNA library obtained from Stratagene Inc., of La Jolla, California was screened using standard techniques. The library was titrated and plated as recommended and filters were prepared. Following prehybridization at 42 °C in 50 % formamide, 5 x SSPE, 5 x Denharts solution, 1 % SDS and 100 ug of salmon sperm DNA/ml, the filters were hybridized at the same temperature and in the same solution containing the denatured radiolabelled probe using 10^5 cpm/ml of hybridization solution. The probe was labelled using a random priming kit (Amersham). After 16 hours the filters were washed in 2 x SSC containing 0.5 % SDS for 2 x 30 mins at 52 °C. The filters were exposed overnight using intensifying screens at -70 °C. Positive clones were rescreened two times until all plaques on a plate were positive. The inserts from several positive clones were subcloned into the plasmid pBluescript SK+ by *in vivo* excision as recommended by the supplier.

Several clones were mapped by restriction enzyme analysis and were found to fall into two distinct groups characterized by the length of a SpeI/BamH1 restriction fragment. The first of these groups comprised three of the restriction mapped clones which each had a 240 bp SpeI/BamH1 restriction fragment. The other group comprised a clone which had a 340 bp SpeI/BamH1 fragment. Analysis of this fragment is described in Example 5.

The three clones which exhibited the 240 bp SpeI/BamH1 restriction fragment were fully or partially sequenced (Sequenase 2.0, U.S. Biochemicals) and the characterization

Nucleotide sequence analyses revealed that two of the cDNA clones were substantially identical, although they differed in length, and one had a mutation. One of the clones was full length and contained an open reading frame encoding 188 amino acid residues in which the first 21 amino acids are a clevable signal sequence. The other of the two substantially identical clones terminated at the G of the start initiation codon. It could be inferred by sequence analysis of additional clones that the sequence preceeding the G reads ACCAT. Both of the clones were found to have the same coding region nucleotide sequence, which is depicted in Figure 3 (SEQ ID NO:4). The figure omits the initial thymine and adenine of the start codon (TAG) which were not present in the isolated clones. The deduced amino acid sequence of the open reading frame of the coding region of both of these two cDNA clones is shown in Figure 4 (SEQ ID NO:5). The resulting protein encoded by this sequence is referred to hereinafter as VEGF-B_{167}. In each of the protein names used herein, the subscript number refers to the number of amino acids in the mature protein without the signal sequence.

As would be expected, a comparison of the amino acid sequence encoded by these two clones with the partial amino acid sequence deduced from cDNA clone of Example 1 showed a striking similarity. However, the two open reading frames in the clone of Example 1, each of which encoded an amino acid sequence homologous to a different portion of VEGF, are both present in the same reading frame in each of these two clones according to Example 2. The frame shift in the clone of Example 1 is caused by an insertion of two extra adenine units which displace the C-terminal part of the clone of Example 1 out of frame. The reason for this is not presently understood, but may be due to a cloning artifact.

The coding part of the third clone had a nucleotide sequence identical to those of the preceding two clones

sequence of this third clone (SEQ ID NO:6). To facilitate identification, the 21 extra bases are underlined in the Figure. This insertion gives rise to 7 additional amino acid residues in the mature protein. Thus the resulting protein encoded by this longer cDNA is termed VEGF-B_{174}. The amino acid sequence of the protein encoded by the cDNA of Figure 5 is depicted in Figure 6 (SEQ ID NO:7). The seven additional amino acids also are underlined in the figure for ease of identification. The additional amino acids are inserted into the sequence in a splice site, and sequencing of mouse genomic DNA clones indicates that these additional amino acids are the result of true alternative splicing. Furthermore, based on what is known about the receptor binding site locations of PDGF, the insertion occurs in a position in the protein which is probably part of a receptor binding site. The insertion is thus likely to affect receptor binding and could be of functional importance in influencing antagonist and/or different receptor specificify.

Example 3: Hybrid cDNA clone.

As previously pointed out this original cDNA clone of Example 1 was not full length and may contain an artifact. However, if the extreme 5' nucleotide sequence of the clones which encode VEGF-B_{167} and/or VEGF-B_{174} is added, the open reading frame encodes a protein of 133 amino acids, yielding a mature protein which is 112 amino acids long and hence is named VEGF-B_{112}. The hybrid cDNA sequence encoding VEGF-$B_{...}$ (SEQ ID NO:8) is shown in Figure 7, and the amino acid sequence of the corresponding protein (SEQ ID NO:9) is illustrated in Figure 8.

Figure 9 shows a multiple amino acid sequence alignment for comparison purposes of the 167 amino acid variant of mouse Vascular Endothelial Growth Factor B (mVEGF-B_{167}), mouse Vascular Endothelial Growth Factor (mVEGF...), human Placenta

A (mPDGF A), and mouse Platelet Derived Growth Factor B (mPDGF B). Amino acid residues identical to mouse VEGF-B_{167} are boxed. The homologous relationship of the sequences is apparent, and the figure clearly demonstrates the conserved structure of the growth factors belonging to the PDGF/VEGF family of growth factors, and that VEGF-B is a structural homolog of the other growth factors of this group. Pairwise comparisons of the amino acid sequences show that mouse VEGF-B is approximately 43% identical to mouse $VEGF_{164}$, approximately 30% identical to human PlGF, and approximately 20% identical to mouse PDGF A and B. The conserved cysteine residues are particularly noteworthy. It can be seen that the first eight cysteine residues in the N-terminal domains (i.e. the PDGF-like domains) of the five growth factors are shared by all members of this family, and it is thus evident that the eight cysteine residues, which are involved in intramolecular and intermolecular disulfide bonding, are invariant among these growth factors. Furthermore, the C-terminal domains of mouse VEGF-B_{167} and $VEGF_{164}$ also display a significant similarity with eight additional conserved cysteine residues and several stretches of basic amino acids.

Example 4: Cloning of human VEGF-B cDNA.

10^6 λ-clones of human fibrosarcoma cDNA library HT1080 in λgt11 (Clontech) were screened with the ≈0.9 kb insert of the mouse VEGF-B clone pcif 2 according to standard procedures. Among several positive clones, one, termed H.1 was analyzed more carefully and its nucleotide sequence was determined. The nucleotide sequence indicated that a 207 amino acid isoform of human VEGF-B was encoded. Analysis of this isoform is described subsequently in Example 6. Based on the H.1 sequence two oligonucleotides were designed that would amplify the whole coding region of putative human cDNA corresponding to mouse VEGF-B 167 isoform:

5'-CACCATGAGCCCTCTGCTCC-3' (forward) (SEQ ID NO:18)

5'-GCCATGTGTCACCTTCGCAG-3' (reverse) (SEQ ID NO:19)

These oligonucleotides were used to amplify by polymerase chain reaction (PCR) the whole coding region of human VEGF-B from oligo-dT primed human erythroleukemia cell (HEL) RNA. The amplified product was cloned into the pCR II-vector of TA cloning kit (Invitrogen) and sequenced using standard techniques. The nucleotide sequence of the human VEGF-B cDNA clone is shown in Fig. 10 (SEQ ID NO:10), and the deduced amino acid sequence of human VEGF-B_{167} is shown in Fig. 11 (SEQ ID NO:11).

The full length mouse cDNA clone of Example 2 and the full length human cDNA clone of Example 4 each encode a polypeptide of 188 amino acids containing an N-terminal hydrophobic putative signal sequence. In analogy with VEGF, the signal peptidase cleavage site is believed to be located between Ala 21 and Pro 22. The putative cleavage site of the signal peptidase is indicated in Figure 16 by an arrow. Accordingly, the processed VEGF-B polypeptides of these two clones each contain 167 amino acids.

Example 5:

The clone which exhibited the 340 bp SpeI/BamHI fragment isolated in Example 2 was analyzed, and the major portion was found to be identical to the first two clones of Example 2 which exhibited the 240 bp SpeI/BamHI fragment. The difference is due to the presence of an insertion in the C-terminal part of the sequence.

This 340 bp SpeI/BamHI DNA fragement encodes a further isoform of mouse VEGF-B containing 207 amino acids. The coding portion of the DNA encoding this protein (SEQ ID NO:12) is illustrated in Figure 12, and the translated amino acid sequence (SEQ ID NO:13) is illustrated in Figure 13. After cleavage of the 21 amino acid leader sequence, the mature protein contains 186 amino acids and is referred to

alternative DNA splicing as described below with reference to Figure 17.

Example 6:

The H.1 clone isolated as described in Example 4 was found to encode a 207 amino acid isoform of human VEGF-B. The coding portion of the DNA (SEQ ID NO:14) encoding this protein is illustrated in Figure 14 and the translated amino acid sequence (SEQ ID NO:15) is illustrated in Figure 15. Again, this isoform, which is designated hVEGF-B_{186}, appears to be a product of alternative splicing.

Both the mVEGF-B_{186} of Example 5 and the hVEGF-B_{186} of Example 6 include a 101 base pair insertion between nucleotides 414 and 415 of the coding sequence of VEGF-B_{167}. Following the insertion, the nucleotide sequences of these cDNA clones were identical to the correponding VEGF-B_{167} sequences. The position of the 101 base pair insertion corresponds to the exon 5-exon 6 junction in VEGF. The insertion results in a frameshift which causes the C-terminal domains of the two VEGF-B isoforms to be entirely different.

The divergence of the C-terminal amino acid sequences starting at amino acid 116 in SEQ ID NOS 11 and 15, which correspond to the two principal VEGF-B isoforms, VEGF-B_{167} and VEGF-B_{186}, is reflected by the different biochemical characteristics of the two isoforms. The C-terminal domain of VEGF-B_{167} is strongly basic (net charge +13) and binds heparin. The C-terminal domain of VEGF-B_{186} is weakly basic (net charge +5) and has a long stretch of hydrophobic amino acid residues in its C-terminus. The hydrophobic tail in VEGF-B_{186} is unlikely to behave as a transmembrane domain since this variant of VEGF-B is secreted from cells. Therefore, despite an identical N-terminal domain, these two principal isoforms of VEGF-B have very different biochemical properties. The absence of the highly basic heparin-binding

from cells. However, the secretion of VEGF-B_{186} is remarkably slow; in a pulse chase experiment using transfected cells, VEGF-B_{186} homodimers were not found in the medium before 1 hour. In contrast, VEGF homodimers and VEGF-B_{186}·VEGF dimers appear in the medium within 30 minutes.

Figure 16 shows the aligned amino acid sequences of mouse and human VEGF-B_{167} and VEGF-B_{186} (SEQ ID NOS:5, 11, 13 & 15) in one-letter code. Identical residues are enclosed in boxes, while amino acid residues which differ between mouse and human VEGF-B_{167} and VEGF-B_{186} isoforms are outside the boxes. Mouse and human VEGF-B display approximately 88 % amino acid sequence identity and are highly basic, especially in their C-terminal regions. The C-terminal domains of murine and human VEGF-B_{186} are approximately 85% identical at the amino acid level. The C-terminal domains of murine and human VEGF-B_{167} are approximately 84% identical at the amino acid level. Both polypeptides lack the consensus sequence for N-linked glycosylation (N-X-T/S). The arrow indicates the putative cleavage site for the signal peptidase between Ala^{21} and Pro^{22}. Excluding the signal sequences, the mouse and human VEGF-B_{167} amino acid sequences are highly homologous with only 20 replacements out the the 167 residues. The replacements are clustered in the N-terminus, in two regions around amino acids 60 and 145. All cysteine residues in both VEGF-B_{167} proteins are invariant, but the eight cysteine residues in the C-terminal end of VEGF-B_{167} are not conserved in the VEGF-B_{186} isoforms. It is notable that the mouse and human sequences in the region between residues 66 and 129 are identical apart from one evolutionarily conserved replacement (Q105R). This is of importance since the receptor binding domains are found within this portion of the protein (compared to PDGF structure). From this it can be concluded that it is likely that mouse and human VEGF-B will exhibit cross-reactive binding on the receptor level and thus display identical or

Example 7: Exon-intron structures of mouse and human VEGF-B genes.

The structure of the human VEGF-B gene was determined by restriction mapping and nucleotide sequence analysis of cloned PCR fragments obtained from PCR reactions employing human genomic DNA as the template, except in the case of the first exon and intron, which were identified from a genomic λ-clone. The structure of the mouse gene was determined by restriction mapping and nucleotide sequence analysis of cloned PCR fragments amplified using different combinations of primers. As a template in these PCR amplifications an isolated genomic λ clone containing the entire mouse VEGF-B gene was used.

Procedure.

Several λ clones for the mouse VEGF-B gene were isolated from a 129/Sw λFIX genomic library as recommended by the supplier (Stratagene, Inc.). The ≈0.9 kb SalI/NotI insert of the pcif2 cDNA for VEGF-B (SEQ ID NO:1) was used as the probe. λ DNA from several positive clones were isolated from plate lysates. One of the positive λ-clones (clone 10) was subcloned as BamH1 fragments into pBluescript SK (Stratagene Inc.). Isolated DNA from this same clone was also used as the template in PCR reactions (100 ng of λ DNA/reaction) and the coding parts of the mouse VEGF-B gene were amplified using different combinations of primers. The nucleotide sequences of these primers were derived from the cDNA clones encoding murine VEGF-B_{167} and murine VEGF-B_{186}. Taq DNA polymerase (2.5 U/reaction) was used. The generated PCR fragments were directly cloned into the TA-cloning vector pCR II (Invitrogen Inc.). The exon-intron structure of the mouse VEGF-B gene was established by nucleotide sequence analysis of the subcloned Bam H1 genomic fragments and of the cloned PCR products.

A human genomic λ-clone was isolated by screening 1 x 10^6 clones of a human genomic library in EMBL-3 SP6/T7

PCR-fragment spanning 5' sequences of human VEGF-B cDNA as the probe. The washing conditions were: one wash at 1 x SSC at room temperature for 30 minutes and two washes at 1 x SSC at 65°C for 30 minutes. Primers for the PCR were:

5'-CACCATGAGCCCTCTGCTCC-3' (forward) (SEQ ID NO:18) and

5'-GGGCATCAGGCTGGGAGACAG-3' (reverse) (SEQ ID NO:19).

The positive λ-clone was subcloned as SacI fragments into pGEM 3Z vector (Promega) and was found to carry the 5'-region of the gene. The remaining parts of the human VEGF-B gene were amplified by PCR using genomic DNA as the template. Different combinations of primers derived from the human cDNA sequence were used. Dynazyme DNA polymerase (2.5 U/reaction, Finnzymes) was used. The amplified PCR fragments were directly cloned into the TA-cloning vector pCR II (Invitrogen Inc.). The exon-intron boundaries and the length of the short introns of the mouse and human VEGF-B genes were determined by nucleotide sequence analysis using vector specific primers or suitable primers derived from the cDNA sequences. The length of the larger introns were calculated based on the length of the amplified PCR fragments when analyzed by agarose gel electrophoresis.

Results.

The results showed that the coding parts of the mouse and human VEGF-B genes span approximately 4 kb of DNA and both genes are divided into seven coding exons ranging from 19 bp (E7) to 236 bp in length (E6). Figure 17 is a schematic representation of the structures of the mouse and human genes for VEGF-B. The exon sizes in base pairs are noted inside the boxes, and the sizes of the introns are noted between the boxes. The introns are not shown to scale. The structures of the untranslated flanking regions of mouse and human VEGF-B genes were not established and are represented by gray boxes. The exon-intron junctions in both genes are listed in the following Table 3:

Table 3

Exon Length	Donor Site	Intron Length (bp)	Acceptor Site
Mouse			
E1 60	T CGC ACC CAG/gtacgtgcgt a Arg Thr Gln	~590	tttcccacag/GCC CCT GTG T Ala Pro Val S
E2 43	CAG AAG AAA G/gtaataatag Gln Lys Lys V	287	ctgcccacag/TG GTG CCA TG al Val Pro Tr
E3 197	C CGA ATG CAG/gtaccagggc l Arg Met Gln	161	ctgagcacag/ATC CTC ATG A Ile Leu Met I
E4 74	GT GAA TGC AG/gtgccagcca ys Glu Cys Ar	178	ctcctcctag/G GTT GCC ATA g Val Ala Ile
mVEGF-B_{186}			
E5 36	AG CCA GAC AG/gtgagttttt ys Pro Asp Ar	~200	ctcctcctag/G GTT GCC ATA g Val Ala Ile
E6A 211	Stop codon in exon 6 (TAG)	---	----------
mVEGF-B_{167}			
E5 36	AG CCA GAC AG/gtgagttttt ys Pro Asp Se	~300	cccactccag/C CCC AGG ATA r Pro Arg Ile
E6B 135	AC ACC TGT AG/gtaaggagtc sp Thr Cys Ar	~2.6 kb	cactccccag/G TGC CGG AAG g Cys Arg Lys
E7 19	Stop codon in exon 7 (TGA)	---	----------
Human			
E1 60	C CCC GCC CAG/gtacgtgcgg a Pro Ala Gln	~760	tctcccacag/GCC CCT GTC T Ala Pro Val S
E2 43	CAG AGG AAA G/gtaatactta Gln Arg Lys V	275	ctgctcccag/TG GTG TCA TG al Val Ser Tr
E3 197	C CGG ATG CAG/gtactgggca l Arg Met Gln	244	ctgagcacag/ATC CTC ATG A Ile Leu Met I
E4 74	GT GAA TGC AG/gtgccagcca ys Glu Cys Ar	~710	tacttttcag/A CCT AAA AAA g Pro Lys Lys
hVEGF-B_{186}			
E5 35	AG CCA GAC AG/gtgagtcttt ys Pro glu Ar	200	tcctccctag/G GCT GCC ACT g Ala Ala Thr
E6A 211	Stop codon in exon 6 (TAG)	---	----------
hVEGF-B_{167}			
E5 36	AG CCA GAC AG/gtgagtcttt ys Pro Glu Se	~300	cccactccag/C CCC AGG CCC r Pro Arg Pro
E6B 135	AC ACC TGC AG/gtaggtttgg sp Thr Cys Ar	736	cctcctcag/G TGC CGG AAG g Cys Arg Lys
E7 19	Stop codon in exon 7 (TGA)	---	----------

As previously stated, exon 6 contains an alternative splice acceptor site which enables the gene to produce two different transcripts for VEGF-B isoforms. VEGF-B_{167} uses exons 1-5, the last part of exon 6, and exon 7 (TGA). VEGF-B_{186} uses exons 1 through 5, the first part of exon 6, and terminates in the last part of exon 6 (TAG). Exon 7 is not translated in VEGF-B_{186} since the insertion of the first part of exon 6 introduces a frame shift and gives rise to a stop codon in the last part of exon 6. The position of the stop codon (TAG) for VEGF-B_{186} is marked in exon 6B, and the stop codon (TGA) for VEGF-B_{167} is marked in exon 7.

The introns in both genes vary from 161 bp to approximately 2.6 kb. The length of each exon and the locations of the splice junctions in the two genes were identical, and all splice donor and acceptor sites follow the canonical GT/AG rules, Padgett et al., *Annual Rev. of Biochemistry*, 55:1119-50 (1986). The only notable difference between the mouse and the human genes are the length of introns 1, 4 and 6 which are longer in the mouse gene. All exon-intron boundaries were found to be conserved between VEGF-B and VEGF, but the introns in the VEGF-B genes were generally smaller than in the VEGF gene.

The 300 bp-intron after the exon 5 in VEGF-B differs from the corresponding one in VEGF, which is 3 kb in length and contains an alternatively spliced exon found in the transcripts for $VEGF_{189}$ and $VEGF_{206}$, encoding many basic amino acid residues. When this intron in VEGF-B was analyzed more carefully, no exon corresponding to the 6th exon of VEGF could be found. Instead, the 3' end of this intron and the following exon were found to be identical with the corresponding sequences of the cDNA clones encoding VEGF-B_{186}. This is explainable by the fact that the mRNA for VEGF-B_{186} is formed by use of an alternative splice acceptor site during mRNA splicing, resulting in an insertion of a 101 bp intron sequence into these mRNAs.

Figure 18 shows a comparative hydrophilicity analysis of murine VEGF-B_{167} and VEGF-B_{186}. The profiles were generated according to Kyle and Dolittle using a window of nine (9) residues. As would be expected, the pattern of hydrophilicity/hydrophobicity is essentially identical from amino acid 1 through amino acid 115. After amino acid 115, the hydrophilicity/hydrophobicity patterns diverge because of the frame shift introduced by the first part of exon 6. Thus, VEGF-B_{167} and VEGF-B_{186} can be expected to exhibit both similar and dissimilar activities.

Figure 19 is a dendrogram showing the phylogenetic relationship of the amino acid sequences of five members of the VEGF/PDGF family of growth factors. The number of replacements or substitutions decreases from the left to the right of the chart. It can be seen that VEGF-B lies between VEGF and the platelet derived growth factor (PDGF) group.

The multiple amino acid sequence alignments of Figures 9 and 16 and the phylogenetic analysis of Figure 19 were carried out accoring to Hein, *Methods in Enzymology*, Vol. 183, pp. 626-45, Academic Press Inc., San Diego (1990) using the PAM 250 distance table.

Example 8: Antibody Production.

a. Antiserum to Mouse VEGF-B.

Antisera to mouse VEGF-B were raised by immunizing rabbits with a 18-mer oligopeptide comprising the N-terminal region of processed VEGF-B, coupled to keyhole limpet hemocyanin. Cysteine residues were introduced as the N-terminal and C-terminal amino acid residues to allow coupling of the peptide to the carrier protein using SPDP (Pharmacia). The sequence of the oligopeptide was

C-P-V-S-Q-F-D-G-P-S-H-Q-K-K-V-V-P-C (SEQ ID NO:21).

Each rabbit received a subcutaneous injection with 300 µg of the peptide conjugate emulsified in Complete Freunds Adjuvant. Subcutaneous booster injections were given every

Incomplete Freunds Adjuvant. Sera were obtained after the second booster injections.

b. Antiserum to Human VEGF-B.

Antipeptide antiserum to human VEGF-B was generated by immunizing rabbits with a branched 23-mer oligopeptide comprising the following N-terminal region amino acid residue sequence (SEQ ID NO:22):

S-Q-P-D-A-P-G-H-Q-R-K-V-V-S-W-I-D-V-Y-T-R-A-T.

The branched 23-mer oligo peptide was synthesized according to Tam, "Synthetic peptide vaccine design: synthesis and properties of a high-density multiple antigenic peptide system", *Proc. Natl. Acad. Sci. USA*, Vol. 85, pages 5409-413 (1988). In the first immunization, rabbits were subcutaneously injected with 500 μg of the branched peptide emulsified in Complete Freunds Adjuvant. In the subsequent boosters, 200 μg of the antigen emulsified in Incomplete Freunds Adjuvant was injected. Antisera were collected after the second and third boosters by conventional techniques.

Example 9: Biochemical properties of VEGF-B_{167}, homodimerization, and heterodimerization with VEGF.

The biochemical properties of human VEGF-B_{167} were examined in transfected human embryonic kidney 293EBNA cells (Invitrogen, Inc.). cDNA inserts encoding human VEGF-B_{167} and human $VEGF_{165}$ [see Keck et al., *Science*, Vol. 246, pages 1309-312 (1989)] were individually cloned into the pREP7 expression vector (Invitrogen, Inc.). Human embryo kidney 293EBNA cells (expressing Epstein-Barr virus nuclear antigen-1) were transfected by transient transfection with the respective expression plasmids using calcium phosphate precipitation, and the cells were incubated for 48 hours. As a control, cells also were transfected with an expression vector containing the VEGF-B_{167} cDNA in reverse orientation. Monolayers of cells were incubated in methionine free and

with 100 μCi/ml [^{35}S]methionine and [^{35}S]cysteine (Promix, Amersham Inc.) in the same medium for 2 hours. The labeling medium was replaced with normal medium without serum, and labelled proteins were chased for 6 hours. Heparin was included during the chase when indicated (100 μg/ml). Media were collected after the chase period, and cells were solubilized in 10 mM Tris pH 7.5, 50 mM NaCl, 0.5% sodium deoxycholate, 0.5% Nonidet P-40, 0.1% SDS and 0.1 U/ml aprotinin.

VEGF-B_{167} was expressed in the cells transfected with the plasmids containing the VEGF-B_{167} DNA. Aliquots of the culture supernatants from cells treated or untreated with heparin and detergent solubilized cell lysates were subjected to immunoprecipitation with the specific antipeptide antiserum to VEGF-B obtained as described in Example 8 and analyzed by SDS-PAGE under reducing conditions unless otherwise indicated. The data show that VEGF-B_{167} homodimers and VEGF-B_{167} - $VEGF_{165}$ heterodimers are released from cells by heparin. By heparin treatment (1-100 μg/ml) or 1.2 M NaCl, VEGF-B_{167} was released from cells and found in the supernatant. If cells were not treated with heparin, VEGF-B_{167} remained cell-associated and was not released into the culture medium. Under the same conditions, $VEGF_{165}$ homodimers are secreted from the cells and found in the culture supernatants without heparin treatment.

Under reducing conditions, human VEGF-B_{167} migrated with a Mr of 21 kDa. Analysis of culture supernatants under non-reducing conditions showed that VEGF-B_{167} migrated as an Mr 42 kDa species indicating a dimeric structure. These results suggest that VEGF-B_{167} forms disulfide-linked dimers associated with the cell surface, probably through ionic interactions with extracellular heparan sulfate proteoglycans. The association is likely to be mediated by the C-terminal basic domain, as observed for the longer splice variants of VEGF.

Since VEGF has been shown to form heterodimers with PlGF, it was decided to test whether $VEGF_{165}$ could also form heterodimers with VEGF-B_{167}. For this purpose 293EBNA cells were co-transfected with expression vectors encoding both human $VEGF_{165}$ and human VEGF-B_{167}, and VEGF-B_{167} was expressed in combination with $VEGF_{165}$. Metabolically labelled proteins were chased in the presence of heparin, and immunoprecipitations were carried out with antisera to either VEGF-B_{167} or $VEGF_{165}$. The antiserum to human VEGF was from R&D Systems. Under non-reducing conditions the VEGF-B_{167}·$VEGF_{165}$ heterodimers migrated as Mr 42-46 kDa species. The results show that VEGF-B can form disulfide linked heterodimers with VEGF, which, in the absence of heparin, remain cell-associated. Since homodimers of $VEGF_{165}$ are efficiently secreted into the media, VEGF-B appears to determine the secretion of the heterodimer.

VEGF-B is synthesized normally in the endoplasmic reticulum of the source cell for subsequent export. Recombinant VEGF-B may be produced by inserting a DNA sequence encoding the VEGF-B protein together with suitable operatively linked promoter and control sequences into a suitable vector, such as the well known plasmid pBR322 or a derivative thereof, transforming or transfecting a suitable host cell, such as *E. coli* or a Cos cell, with the resulting vector or other systems well known in the art, screening the resulting transformants or transfectants for VEGF-B expression, and then culturing cell lines or bacterial cell strains which are positive for the expression of VEGF-B. Either a eukaryotic vector or a prokaryotic vector may be used, depending on the type of cell which is to be transfected or transformed therewith. A particularly preferred system for production of recombinant VEGF-B is the baculovirus - insect cell system, which has proved capable of producing excellent yields of recombinant protein.

Example 10: VEGF-B expression using the baculovirus system.

10.1 VEGF-B with its own signal peptide.

a) Cloning and Transfection.

The complete human VEGF-B_{167} gene was inserted into a commercially available plasmid pCRII (Invitrogen Corp.). The HindIII-XbaI fragment from the resulting plasmid pCRII-VEGF-B_{167}, which encodes the whole open reading frame of VEGF-B_{167} then was cloned into pFASTBAC1, and both the 3'- and 5'-junctions were sequenced. Bacmid-DNA was prepared according to the manufacturers instructions for the "Bac-To-Bac™ Baculovirus Expression System" (Life Technologies Inc.) and lipofected to Sf900II-adapted Sf9 cells (obtained from Dr. Christian Oker-Blom). Sf9 cells are from the American Type Culture Collection Cell Repository Line Bank, Rockville MD (ATCC CRL-1711). The transfected cells were then cultured on standard TMN-FH medium in 25 cm^2 culture dishes.

b) Assay for protein expression.

About 72 hours after transfection, the cells were lysed and 1 ml of culture supernatant and the cell lysate were assayed for expressed VEGF-B by immunoprecipitation as described in Example 9 and Western blotting. Lysates from three out of four independently transfected cell cultures were found positive for VEGF-B, although the intensity of the signal in the Western blot varied. The expressed VEGF-B polypeptide in each case was found to correspond in size to the protein expressed in mammalian cells in Example 9.

The viral stock from the cells that gave the strongest signal in Western blotting was amplified two rounds by infecting cells and collecting new virus from the medium. The resulting supernatant was analyzed. Uninfected cells were also analyzed as a negative control. Time course analysis showed that cells harvested between 48 and 72 hours after infection contained the greatest amount of VEGF-B. After 96 hours post infection, as a result of virus-induced cell lysis, VEGF-B could also be detected in the culture

Recombinant VEGF-B could be precipitated from the lysate between 20% and 40% $(NH_4)_2SO_4$.

10.2 VEGF-B withthe Melittin signal peptide (pVTBac).

a) Cloning and transfection.

A polymerase chain reaction (PCR) fragment from nucleotide position 68 to 141 was used to introduce a BamHI restriction site immediately after the signal cleavage site in the plasmid pCRII-VEGF-B_{167} from Example 10.1. The BamHI fragment from this modified pCRII-VEGF-B_{167} construct was cloned into BamHI opened pVTBac [Tessier et al., "Enhanced secretion from insect cells of a foreign protein fused to the honeybee mellitin signal peptide", *Gene*, Vol. 98, page 177 (1991)]. Both 3'- and 5'-junctions were sequenced. Sf9 cells were cotransfected with the aforedescribed pVTBac vector which contained the human VGEF-B_{167} gene, and with linearized baculovirus DNA (Insectin™, Invitrogen Corp.). The transfected cells then were cultured in TMN-FH medium.

b) Assay for protein expression.

Forty-eight hours after transfection, the supernatant was collected and subjected to primary screening by immunoprecipitation. Four positive plaques were isolated.

10.3 A cDNA insert encoding murine VEGF-B_{186} (EcoR1 cut cDNA fragment from a murine VEGF-B_{186} cDNA (SEQ ID NO:12) clone) was cloned into pFASTBAC 1. An EcoR1 cut cDNA fragment from murine VEGF-B_{167} (SEQ ID NO:4) was also cloned into pFASTBAC 1. The resulting plasmids were transformed into bacteria as described in 10.1 above, and recombined plasmids were isolated and lipofected into Sf9 and Sf21 cells. Supernatants containing recombinant baculovirus were amplified by several rounds of reinfection of Sf21 cells. The final titers of the baculovirus stocks were determined by plaque titration and found to vary between 4 x 10^4 and 2 x 10^9 baculovirus particles per milliliter of stock supernatant.

Example 11: Large Scale Production of Recombinant VEGF-B.

Sf21 cells [see Vaughn et al., *In Vitro*, 13:213-17 (1977)] were infected with the baculovirus stocks of Example 10 at a multiplicity of infection of 10 virus particles per cell. The infected Sf21 cells were grown in roller flasks and seeded at a density of 2 x 10^6 cells per ml of serum free medium (Sf900II, Gibco-BRL) for 96 hours. Culture media and cells were then harvested. Aliquots of the cell lysates and of the media were analyzed by SDS-PAGE. Total protein patterns were visualized by staining the gels with Coomassie Brilliant Blue and the presence of expressed VEGF-B isoforms were visualized by immunoblotting using specific antipeptide antibodies to human and mouse VEGF-B as described above in Example 8. The analysis revealed that both human and mouse VEGF-B_{167} polypeptides were of the expected sizes of 21.5 kDa. Both proteins were retained intracellularly in the infected cells and not released into the medium. In contrast, mouse VEGF-B_{186} was readily secreted into the medium in a dimeric form. The VEGF-B_{186} homodimers migrated as a 52-54 kDa species which suggested that insect cell produced protein did not undergo the same covalent modification as found for VEGF-B_{186} secreted from transfected Cos-1 cells.

Example 12: Transfection and analysis of Cos-1 cells expressing VEGF-B_{186}.

cDNA inserts encoding mouse VEGF-B_{186} and human $VEGF_{165}$ were cloned into the pSG5 expression vector [Green et al., *Nucleic Acid Res.*, 16:369 (1988)]. Cos-1 cells were maintained in minimal essential medium (MEM) containing 10% fetal calf serum, 2mM glutamine and appropriate antibiotics. For transfections, the cells were replated into 90 mm Petri dishes. The cells were transfected with the expression vectors, separately or in combination, using calcium phosphate precipitation and incubated for 36-48 hours. Monolayers of cells were incubated in medium free of

same medium containing 100 μCi/ml of [^{35}S]-methionine and [^{35}S]-cysteine for 2 hours (Promix Amersham Inc.).

For the pulse-chase experiments, the cells were labeled for 30 minutes, washed twice with normal medium and then incubated for up to 6 hours in MEM without fetal calf serum. Media were collected after the chase period and the cells were solubilized in 10 mM Tris buffer pH 7.5 containing 50 mM NaCl, 0.5% deoxycholate, 0.5% nonidet P-40 and 0.1% SDS. Aliquots of the media and the cell lysates were subjected to immunoprecipitation using the specific antiserum to mouse VEGF-B from Example 8a and a specific antiserum to human VEGF commercially available from R&D Systems. The precipitates were analyzed by SDS-PAGE.

Example 13: Biochemical properties of VEGF-B_{186} expressed in transfected Cos-1 cells.

The biochemical properties of mouse VEGF-B_{186} were examined in Cos-1 cells transiently transfected as described in Example 12 with an appropriate expression vector. The cells were metabolically labelled, and proteins from the labelled cells were immunoprecipitated using an antipeptide antibody to VEGF-B. The precipitated material was subjected to SDS-PAGE analysis under reducing conditions. Both the cell culture medium (M) and a detergent solubilized cell lysate (L) were analyzed. The results are shown in Figure 22. It can be seen that cell associated VEGF-B_{186} migrated as an approximately M_r 24,000 polypeptide under reducing conditions. In contrast, VEGF-B_{186} present in the medium of transfected cells migrated as a M_r 32,000 species, suggesting that the protein was covalently modified during its intracellular transport and secretion. The corresponding molecules were not detected in cell lysates or media from mock transfected Cos-1 cells used as a control.

Immunoprecipitation of media and SDS-PAGE analysis under non-reducing conditions, showed an approximately M_r

linked homodimers. Including 100 ug/ml of heparin during the labelling did not affect the secretion or release of VEGF-B_{186} homodimers from the transfected cells.

Example 14: Biosynthesis of VEGF-B_{186} homodimers.

The biosynthesis of VEGF-B_{186} homodimers was examined by pulse-chase experiments. Transfected Cos-1 cells were metabolically labelled for 30 minutes and then chased for up to 4 hours. Immunoprecipitation and SDS-PAGE analysis of detergent solubilized cell lysates and media showed that the cell-associated approximately M_r 24,000 species was readily detected in the lysates throughout the chase period. The decrease in the intensity of this molecular species was associated with an increase in the M_r 32,000 protein present in the media. The M_r 32,000 species appeared in the medium after 1 hour of chase. Highest levels of secreted VEGF-B_{186} were obtained after the 4 hour chase period. No intermediates were detected in the cell lysates, but the secreted M_r 32,000 protein appeared slightly heterogenous. The nature of the modification is presently unknown, but N-linked glycosylation can be excluded in the absence of consensus sites for this modification.

Example 15: Formation of heterodimers by VEGF-B_{186}.

As noted above, VEGF-B and VEGF are coexpressed in many tissues and VEGF-B_{167} $VEGF_{165}$ heterodimers are readily formed when coexpressed in transfected cells. To examine whether VEGF-B_{186} also could form heterodimers with $VEGF_{165}$, Cos-1 cells were transfected as described above with the appropriate expression vectors, either alone or in combination. Metabolically labelled proteins present in the media from the transfected cells were subjected to immunoprecipitations using antisera to VEGF-B and VEGF. Figure 23A shows the results of SDS-PAGE analysis under reducing conditions of the immunoprecipitates from the cell

separately expressing VEGF-B_{186} and VEGF, respectively. It can be seen that the antisera were specific for VEGF-B and VEGF, respectively, with no detectable cross-reactivity.

Cos-1 cells were cotransfected with expression vectors for VEGF-B_{186} and $VEGF_{165}$. Cell culture media (M) and detergent solubilized lysates (L) from the resulting cells which coexpressed VEGF-B_{186} and $VEGF_{165}$ were subjected to immunoprecipitation and SDS-PAGE analysis under reducing conditions. The results are shown in Figure 23B. The test showed that murine VEGF-B_{186} and human $VEGF_{165}$ form intracellular and secreted heterodimers.

Culture media from cells expressing murine VEGF-B_{186} and human $VEGF_{165}$, either separately or in combination, were subjected to immunoprecipitation using antibodies to VEGF-B and VEGF and analyzed by SDS-PAGE under non-reducing conditions. As a control, cell culture medium from mock transfected cells was analyzed. The results are shown in Figure 23C. It was found that VEGF-B_{186} forms secreted disulfide-linked homodimers and that VEGF-B_{186} and $VEGF_{165}$ together form secreted disulfide-linked heterodimers.

To analyze whether heterodimer formation with VEGF affected the secretion and release of VEGF-B_{186}, pulse-chase experiments were carried out using Cos-1 cells transiently transfected with expression vectors for VEGF-B_{186} and $VEGF_{165}$. Cell associated disulfide-linked heterodimers could be recovered following the 30 minute labelling period, and secreted heterodimers were recovered from the medium already after a 30 minute chase period. The secreted heterodimers accumulated in the medium for up to 2 hours post labelling. In the 4 hour chase time point there was a decrease in the amount of heterodimers in the medium, possibly due to the degradation of the complex. Some VEGF-B_{186}:VEGF heterodimers remained cell-associated throughout the chase. These results suggested that heterodimer formation with VEGF promoted the secretion of VEGF-B_{186} compared to the secretion

heterodimers already following the 30 minute labelling period suggested that the slow release of VEGF-B_{186} homodimers was not due an impaired ability of VEGF-B_{186} to dimerize.

Example 16: Purification of Secreted VEGF-B_{186} Homodimers.

Secreted VEGF-B_{186} homodimers were isolated from serum free culture media of baculovirus infected Sf21 cells as follows:

a. Initial Separation.

The major contaminating protein in the culture media was the baculovirus protein gp64/67, an acidic protein secreted by baculovirus infected cells. To remove this protein, the culture media was concentrated twenty-fold by ultrafiltration and then passed over a Sephadex G-25 column equilibrated in 20 mM phosphate buffer pH 6.5 containing 20 mM NaCL. Eluted proteins were then passed over a CM-Sepharose (Pharmacia) ion-exchange column equilibrated in the same buffer. The column was washed with the phosphate buffer to remove unbound proteins, and bound proteins were eluted by stepwise increasing the NaCl concentration of the elution buffer. The major gp64/67 baculovirus encoded protein did not bind to the ion-exchange column under those conditions while VEGF-B_{186} homodimers eluted at a NaCl concentration of 90 mM. As judged by SDS-PAGE analysis of the eluted fraction, VEGF-B_{186} homodimers were 5-15% pure by this procedure.

b. Purification to Homogeniety.

The VEGF-B_{186} homodimers are purified to homogeneity on a MonoS column coupled to a FLPC system (Pharmacia). Bound protein is eluted with a linear gradient of NaCl in 20 mM phosphate buffer pH 6.5.

Example 17:

In order to find out whether the two VEGF-B splice isoforms exhibited a differential tissue distribution and

carried out using total RNA extracted from mouse brain, heart, liver and kidney and from human embryonic heart and skeletal muscle. The transcripts were analyzed by PCR using four pairs of specific primers covering exons 4 to 7 and exons 3 to 7 in the mouse and human VEGF-B genes, respectively.

Procedure.

Total RNA from mouse and human tissues were isolated using standard procedures as disclosed by Chirgwin et al., *Biochemistry*, 18:5294-99 (1979). Two to five μg of total RNA per reaction were used for first strand cDNA synthesis using avian myelostosis virus reverse transcriptase (20 U/reaction). The reactions were primed with oligo-$(dT)_{15}$. Aliquots of these reactions were used as templates in PCR reactions using *Taq* DNA polymerase (2.5 U/reaction). To amplify mouse cDNA, two pairs of primers were used. These pairs were obtained by combining a common 5'-primer

5'-CACAGCCAATGTGAATGCA (forward) (SEQ ID NO:23),

located in exon 4 with two different 3'-primers 5'-GCTCTAAGCCCCGCCCTTGGCAATGGAGGAA (reverse) (SEQ ID NO:24) and 5'-ACGTAGATCTTCACTTTCGCGGCTTCCG (reverse) (SEQ ID NO:25) (this last primer has a Bgl II site and 4 extra bases in the 5' end) located in exons 6B and 7, respectively. Following analysis by agarose gel electrophoresis, the amplified bands were transferred onto a nylon filter (Genescreen Plus) and sequentially hydbridized with oligonucleotide probes specific for exons 6A and 6B. The oligonucleotide probes were 5'-CTCTGTTCCGGGCTGGGACTCTA (exon 6A) (SEQ ID NO:26) and 5'-TCAGGGCGTTGACGGCGCTGGGTGCAA (exon 6B) (SEQ ID NO:27). The oligonucleotide probes were labeled with [^{32}P]dCTP using terminal transferase to high specific activity. Hybridizations, using 1 x 10^6 cpm of labeled probe/ml of solution, were carried out at 37° C in 6 x SSC containing 5 X Denhardt's solution, 0.5% SDS and 100 μg/ml of salmon sperm DNA. The filters were washed at the same [illegible]

in 6 x SSC containing 0.5% SDS for 2 x 15 min and exposed to film.

The two pairs of primers used for amplification of human cDNA were combined using two different 5'-primers,

5'-CCTGACGATGGCCTGGAGTGT (forward) (SEQ ID NO:28), located in exon 3 and

5'-TGTCCCTGGAAGAACACAGCC (forward) (SEQ ID NO:29), located in exon 4, with a common 3'-primer,

5'-GCCATGTGTCACCTTCGCAG (reverse) (SEQ ID NO:19) located in exon 7. Aliquots of the amplified products were analyzed by agarose gel electrophoresis. The aliquots were directly cloned in the TA-cloning vector pCR II (Invitrogen, Inc.), and generated plasmids were analyzed by nucleotide sequencing. Amplification of GAPDH served as a control.

Results.

Analysis of amplified PCR products by agarose gel electrophoresis showed two major bands of 215 and 316 bp, respectively. These sizes are consistent with the two mRNAs corresponding to VEGF-B_{167} and VEGF-B_{186}. These two bands were of the same intensity suggesting that the two isoforms were expressed at approximately equal levels in all mouse and human tissues examined.

To verify the identity of the amplified products from mouse tissues, the PCR-amplified DNA was transferred to a filter and probed with specific oligonucleotide probes for exons 6A and 6B, respectively. The autoradiograms showed that an exon 6-specific probe hybridized with the 316 bp band while the exon 6B specific probe hybridized with both the 215 bp and the 316 bp amplified bands. These results are consistent with the alternative usage of acceptor site in exon 6 to create the two isoforms of VEGF-B and thus all the amplified products corresponded to those predicted from the sequences of VEGF-B_{167} and VEGF-B_{186} isoforms.

Agarose gel electrophoresis of products of PCR analysis of total RNA isolated from human embryonic heart and muscle

Taken together, these data demonstrate that VEGF-B_{167} and VEGF-B_{186} are the two major isoforms of VEGF-B in tissues. The pattern of the PCR products and the location of the primers indicate that if any still longer splice isoforms exist for VEGF-B, such transcripts use a splice acceptor site located a little more 5' than in the case of VEGF-B_{186}. Furthermore, PCR products corresponding to $VEGF_{121}$, which lacks heparin binding domains, i.e. sequences corresponding to exon 6 in VEGF-B, were not detected. However, splicing of exon 5 to exon 7 would give rise to a transcript encoding an isoform of VEGF-B corresponding to $VEGF_{121}$, and this putative isoform of VEGF-B might be expressed in tissues other than those analyzed in this example.

Example 18: Stimulation of Cell Proliferation.

The ability of VEGF-B_{167} to stimulate endothelial cell proliferation was established through analysis of [^{3}H]thymidine incorporation in human umbilical vein endothelial cells (HUVEC) and in bovine capillary endothelial (BCE) cells.

293EBNA cells were transfected as described above with expression vectors for VEGF-B_{167}, $VEGF_{165}$ or empty vector (mock) in the presence of 1 μg/ml heparin. Conditioned media from these cells were diluted in respective media, applied to human umbilical vein endothelial cells (HUVEC) and to bovine capillary endothelial (BCE) cells and incorporation of [^{3}H]thymidine was measured. As a positive control recombinant bFGF was added to BCE cells.

To elaborate, conditioned media containing human VEGF-B and human $VEGF_{165}$ were collected from 293EBNA cells transfected with the appropriate expression vectors or with empty vector (mock) in the presence of heparin (1 μg/ml) 48 hours posttransfection. Second passage HUVEC were plated into 96-well plates (4 x 10^3 cells/well) in M-199 medium supplemented with 10% fetal bovine serum and incubated for

medium, and cells were stimulated for 48 hours. Fresh conditioned media containing 10 μCi/ml of [^{3}H]thymidine (Amersham Inc.) were added to the cells, and stimulations were continued for another 48 hours. Cells were washed with PBS and trypsinized, and incorporated radioactivity was determined by liquid scintillation counting. BCE cells were seeded into 24-well plates and grown until confluence in minimal essential medium (MEM) supplemented with 10% fetal calf serum. Cells were starved in MEM supplemented with 3% fetal calf serum for 72 hours, after which conditioned media diluted into serum-free medium were added to the cells and the cells were stimulated for 24 hours. [^{3}H]Thymidine was included during the last 4 hours of the stimulation (1 μCi/ml). Stimulations with bFGF were carried out as above using 6 ng/ml of recombinant bFGF (Synergen Inc.). Cells were washed with PBS, lysed with NaOH, and incorporated radioactivity was determined by liquid scintillation counting.

Figure 20 is a bar graph showing fold of induction of [^{3}H]thymidine incorporation by VEGF-B_{167} in human umbilical vein endothelial cells (HUVEC) and in bovine capillary endothelial (BCE) cells, as compared to basal activity induced by conditioned medium from the mock transfected cells. For comparison purposes, the induction by $VEGF_{165}$ and by bFGF are also shown. The bars show the mean ± standard deviation of parallel samples. Similar results were obtained in several other independent experiments. The test results clearly show that VEGF-B induced [^{3}H]thymidine incorporation in both HUVEC and BCE cells and stimulated proliferation of endothelial cells in vitro, thereby demonstrating that VEGF-B is an endothelial growth factor.

Example 19: Identification of human VEGF-B promoter DNA clones and activity.

A human genomic DNA library in bacteriophage λ EMBL was

from the VEGF-B first and second exons as a probe. Two positive clones were obtained, and one of these was subcloned in the Bluescript SKII plasmid as Sac I fragments. A 1.4 kb fragment was obtained, which contained about 0.4 kb of sequences upstream from an Nco I site present in the cDNA (located less than 100 bp upstream of the ATG translational initiation site).

In addition, an XhoI fragment of about 6 kb from the other λ clone was subcloned into the pGEMEX plasmid. This subclone contained about 1.5 kb of sequences upstream from the NcoI site. The SacI/NcoI fragment and an EcoRI (polylinker) - NcoI fragment were subcloned into pGL3 basic vector (Promega) in the respective transcriptional orientation. DNA of these subclones, and from the pGL3 control vector containing the SV40 promoter, was transfected into HeLa cells using calcium phosphate precipitation. Two days after transfection, the luciferase activities were measured from lysates of the transfected cells. The results indicated that the 400 bp SacI/NcoI fragment has promoter activity equal to about 30% of the activity of the pGL3 control vector, while the 1.5 kb fragment gave only background activity. Use of a stronger or more active promoter, for example the CMV promoter or the elongation factor 1-alpha promoter, would probably give higher activity in human cells and tissues. The structure of the cloned fragments is illustrated in Figure 24.

The 1.5 kb fragment upstream of the Nco I site was sequenced. The resulting sequence (SEQ ID NO:17) is illustrated in Figure 25. The sequence obtained revealed a putative silencer element [Weissman and Singer, *Molecular and Cellular Biology*, 11:4228-234 (1991)] composed of two eight-base pair stretches between nucleotides 166-187 (boxed in the drawing). This silencer may be responsible for the relative lack of activity of the 1.5 kb fragment.

Example 20: Analysis of VEGF-B mRNA in melanomas, normal skin and muscle by RT-PCR.

Normal skin and melanoma tissues were obtained from patients attending the Department of Radiotherapy and Oncology, Helsinki University Central Hospital. Four metastatic melanoma specimens were obtained freshly after surgical excision, immediately embedded in Tissue-tek (Miles) and frozen in liquid nitrogen. Samples of normal skin were obtained from volunteer patients undergoing surgery for mammary carcinoma and excision of a cutaneous naevus. All specimens were inspected by a pathologist to confirm the diagnosis.

Total RNA was isolated by the guanidium isothiocyante procedure [Chomczynski et al., *Anal. Biochem.* 162:156-159 (1987)]. cDNA was synthesized using 0.2 μg of random hexadeoxynucleotide primers, 5 units of murine reverse transcriptase, 5 μg of total RNA as a template and a first-strand cDNA synthesis kit (Pharmacia). After incubation at 37°C for 1 hour, the reaction mixture was stored at −70°C. Negative control samples for PCR amplification were prepared similarly except that reverse transcriptase was not added. ß-actin also was tested as an internal standard because it is expressed at a constitutive high level, and its expression does not show much variation in different cells.

For PCR amplification, the primer sequences were selected from the VEGF-B and ß-actin genes as follows:
VEGF-B sense: 5'-GCCATGTGTCACCTTCGCAG-3' (SEQ ID NO:19)
VEGF-B antisense: 5'-TGTCCCTGGAAGAACACAGCC-3' (SEQ ID NO:29)
ß-actin sense: 5'-CGGGAAATCGTGCGTGACAT-3' (SEQ ID NO:30)
ß-actin antisense: 5'-GGAGTTGAAGGTAGTTTCGTG-3' (SEQ ID NO:31)
[ß-actin sequences comprise nucleotides 2105-2125 and 2411-2432 from Ng et al., *Mol. Cell Biol.* 5:2720-732 (1985)].
An aliquot of 4 μl from the cDNA reaction product was heated to 94°C for 5 minutes and used as a template for PCR amplification with 20 pmol of primers, 10x PCR buffer, 1.5

was adjusted to 100 µl with DEPC treated water. Denaturation was at 95°C for 1 minute, annealing at 62°C for 45 seconds, and polymerization at 72°C for 50 seconds, for a total of 35 cycles for VEGF-B and 25 cycles for ß-actin. After every 5 cycles, 15 µl aliquots were taken for analysis.

Electrophoresis of 5 µl of the PCR reaction mix was performed in a 2% agarose gel containing ethidium bromide. The size marker DNA fragments ranged in length from 24 to 726 base pairs (ΦX174 DNA/Hinf I marker from Promega, Madison, WI, USA). The tested samples thus included four metastatic melanomas, muscle, normal skin, a negative control (without reverse transcriptase), and the ΦX174 DNA/Hinf I size marker. The results of the RT-PCR analysis for VEGF-B (PCR product lengths 323 and 234 bp) and for ß-actin show that VEGF-B is highly expressed in all melanomas studied, at levels approximately similar to the expression in muscle tissue. On the other hand, normal skin has very little of the VEGF-B RNA. Similar conclusions can be drawn from Northern blotting and hybridization analysis.

The foregoing results indicate that that VEGF-B is a novel growth factor for endothelial cells which plays a role in vascularization, in particular of muscle. Collateral artery growth in ischemic heart or limb may be promoted by arterial administration of a VEGF-B bolus using techniques described by Takeshita et al., *Am. J. Pathol.*, 147:1649-60 (1995). The cell-association of VEGF-B may have several implications for regulation of vascularization and endothelial cell growth. In developing embryos and in contractile tissues, cell-associated VEGF-B may provide spatial cues to outgrowing endothelial cells during establishment and maintenance of the vascular tree. It could also, through its cell-association, support the regeneration of damaged endothelium in adult vessels. Reendothelialization of arterial injury may be promoted by

Asahara et al., *Circulation*, 91(11):2793-802 (1995). The ability of VEGF-B to modulate the secretion of VEGF by heterodimer formation suggests an indirect role of VEGF-B in VEGF signalling, thereby regulating receptor binding and/or activation as described by Potgens et al., *J. Biol. Chem.*, 269(52):32879-85 (1994). The formation of multiple heterodimeric complexes of these growth factors could provide a basis for a diverse array of regulatory signals for endothelial cells.

VEGF-B can be used as a growth factor for populations of endothelial cells *in vitro*. VEGF-B may be used to promote desirable angiogenesis, i.e. the formation of new blood vessels and capillaries; see Takeshita et al., *supra*. For example, it may be useful in promoting the development of the corpus luteum and endometrium as an aid to initiating and/or maintaining pregnancy. It would also be useful in bone repair by virtue of its action on endothelial cells. Administration of VEGF-B may also be useful in supporting embryogenesis, as well as somatic growth and vascular development and differentiation. Topical application of VEGF-B to wounds may be useful in promoting wound healing, and oral administration of VEGF-B may be useful to accelerate the healing of gastric and/or duodenal ulcers. The ability of VEGF-B to modulate the secretion of VEGF by heterodimer formation could provide a therapeutic role for VEGF-B in diseases where VEGF agonists would be useful; see Potgens et al., *supra*.

VEGF-B may exert proliferative effects on mesodermal cells either directly or via improvements in the blood supply.

VEGF-B has been found to be overexpressed in tumors, such as melanomas. Consequently, assays for VEGF-B expression can be used as tools in tumor diagnosis, and suppression of VEGF-B expression, for example with monoclonal antibodies, may be useful to retard tumor growth.

Tumor assays for VEGF-B may be useful as indicators of metastatic risk. For example, use of VEGF-B antibodies analogous to the procedures described by Takahashi et al., *Cancer Res.*, 55:3964-68 (1995) in order to quantitate neovascularization and proliferation could be used as an indicator of metastatic risk from colon cancer. Assays of VEGF-B in body fluids or the tumor itself by histochemistry may be useful as a tumor prognostic factor. An ELISA analogous to the procedure described by Kondo et al., *Biochemica et Biophysica Acta*, 1221(2):211-14 (1994) may be useful to detect VEGF-B upregulation as a tumor screen. An enzyme linked immunoabsorbent assay of VEGF-B expression using techniques described by Boocock et al., *J. Natl. Cancer Inst.*, 87:506-16 (1995) may be useful as a diagnostic index of ovarian cancer. An assay of VEGF-B expression similar to the VEGF assay described by Weindel et al., *Neurosurgery*, 35:439-48 (1994) may be useful as an indicator of malignancy in brain tumors.

Furthermore, because tumor growth requires angiogenesis, administration of VEGF-B may also be useful in promoting tumor growth in laboratory animals in order to test anti-tumorigenic drugs. VEGF-B may also be useful to increase the microvascularity of hypoxic areas of tumors and make them more sensitive to radiation, radiation sensitizing drugs, etc.

The angiogenic action of VEGF-B may be useful in treating ischemic conditions. Administration of an intra-arterial bolus of VEGF-B by the techniques described in Bauters et al., *American Journal of Physiology*, 267(4 Pt 2):H1263-71 (1994) may be useful to treat lower extremity ischemia and increase perfusion in the extremities. Using procedures described by Mesri et al., *Circulation Research*, 76:161-67 (1995) an angiogenic response may be produced in tissue injected with fibroblast cells transduced with a virus which expresses VGEF-B in order to treat tissue

could be used to stimulate the development of collateral circulation in cases of arterial and/or venous obstruction, e.g. myocardial infarcts, ischaemic limbs, deep venous thrombisis, and/or postpartum vascular problems; see Takeshita et al, *supra*.

A VEGF-B/VEGF-B receptor system may be used as an assay system to detect small molecules as agonists/antagonists for development as new drugs. Examples of small molecules which could be detected include, but are not limited to, organic chemicals, peptides, and RNA molecules.

Pharmaceutical compositions may be produced by admixing a pharmaceutically effective amount of VEGF-B protein with one or more suitable carriers or adjuvants such as water, mineral oil, polyethylene glycol, starch, talcum, lactose, thickeners, stabilizers, suspending agents, etc. Such compositions may be in the form of solutions, suspensions, tablets, capsules, creams, salves, ointments, or other conventional forms.

As demonstrated in Example 7, VEGF-B protein also can be used to produce antibodies. In general, conventional antibody production techniques may be used to produce VEGF-B antibodies. For example, specific monoclonal antibodies may be produced via immunization of fusion proteins obtained by recombinant DNA expression.

Labelled monoclonal antibodies, in particular, should be useful in screening for conditions associated with abnormal levels of VEGF-B in the body. For example, an assay of VEGF-B in synovial fluids and/or joint tissue by immunofluorometric techniques analogous to the the procedure described by Fava et al., *Journal of Experimental Medicine*, 180:341-46 (1994) may be useful as a diagnostic indicator of rheumatoid arthritis. A radioimmunoassay of VEGF-B in occular fluid using techniques described by Aiello et al., in *New England Journal of Medicine*, 331(22):1480-87 (1994) may be useful as a diagnostic indicator of diabetic

occlusion. Immunoassays of VEGF-B levels in blood, urine or other bodily fluids may be useful also as a tumor marker; see Kondo et al., *supra*. These monoclonal antibodies to VEGF-B also may be useful in inhibiting angiogenesis associated with high levels of VEGF-B in the body, e.g. in rapidly proliferating, angiogenesis-dependent tumors in mammals, and thereby may retard the growth of such tumors. Treatment with a monoclonal antibody specific for VEGF-B using techniques analogous to those described by Kim et al., in *Nature*, 362(6243):841-44 (1993) may be useful to suppress or inhibit tumor growth *in vivo*. Intravenous and/or subcutaneous injection of monoclonal antibodies to VEGF-B using procedures like those described by Asano et al., in *Cancer Research*, 55:5296-5301 (1995) may be useful to inhibit neovascularization and primary and metastatic growth of solid tumors. For the therapy of humans, chiaserization or humanization of such monoclonal antibodies is to be preferred. Treatment may be effected, e.g., by twice weekly intraperitoneal injection of 10 to 500 μg, preferably 50-100 μg of monoclonal antibody.

VEGF-B antagonists such as antibodies also may be useful to inhibit new blood vessels in diabetic retinopathy, psoriasis, arthopathies and/or vascular tumors such as haemangiomas; see Aiello et al., *supra*.

The foregoing description and examples have been set forth merely to illustrate the invention and are not intended to be limiting. Since modifications of the disclosed embodiments incorporating the spirit and substance of the invention may occur to persons skilled in the art, the invention should be construed to include everything within the scope of the appended claims and equivalents thereof.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: ERIKSSON, Ulf
OLOFSSON, Birgitta
ALITALO, Kari
PAJUSOLA, Katri

(ii) TITLE OF INVENTION: VASCULAR ENDOTHELIAL GROWTH FACTOR-B AND DNA CODING THEREFOR

(iii) NUMBER OF SEQUENCES: 31

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Evenson, McKeown, Edwards & Lenahan
(B) STREET: 1200 G Street, N.W., Suite 700
(C) CITY: Washington
(D) STATE: DC
(E) COUNTRY: US
(F) ZIP: 20005

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US
(B) FILING DATE: 01-MAR-1996

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/397,651
(B) FILING DATE: 01-MAR-1995

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/469,427
(B) FILING DATE: 06-JUN-1995

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/569,063
(B) FILING DATE: 06-DEC-1995

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: EVANS, Joseph D
(B) REGISTRATION NUMBER: 26,269
(C) REFERENCE/DOCKET NUMBER: 1064/41979PCT

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (202) 628-8800
(B) TELEFAX: (202) 628-8844

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 886 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(F) TISSUE TYPE: mouse embryo

(vii) IMMEDIATE SOURCE:
(B) CLONE: pcif2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CGGGACGCCC AGTGGTGCCA TGGATAGACG TTTATGCACG TGCCACATGC CAGCCCAGGG 60

AGGTGGTGGT GCCTCTGAGC ATGGAACTCA TGGGCAATGT GGTCAAACAA CTAGTGCCCA 120

GCTGTGTGAC TGTGCAGCGC TGTGGTGGCT GCTGCCCTGA CGATGGCCTG GAATGTGTGC 180

CCACTGGGCA ACACCAAGTC CGAATGCAGA TCCTCATGAT CCAGTACCCG AGCAGTCAGC 240

TGGGGGAGAT GTCCCTGGAA GAACACAGCC AATGTGAATG CAGACCAAAA AAAAAAAGGA 300

GAGTGCTGTG AAGCCAGACA GCCCCAGGAT CCTCTGCCCG CCTTGCACCC AGCGCCGTCA 360

ACGCCCTGAC CCCCGGACCT GCCGCTGCCG CTGCAGACGC CGCCGCTTCC TCCATTGCCA 420

AGGGCGGGGC TTAGAGCTCA ACCCAGACAC CTGTAGGTGC CGCAAGCCGC GAAAGTGACA 480

AGCTGCTTTC CAGACTCCAC GGGCCCGGCT GCTTTTATGG CCCTGCTTCA CAGGGACGAA 540

GAGTGGAGCA CAGGCAAACC TCCTCAGTCT GGGAGGTCAC TGCCCCAGGA CCTGGACCTT 600

TTAGAGAGCT CTCTCGCCAT CTTTTATCTC CCAGAGCTGC CATCTAACAA TTGTCAAGGA 660

ACCTCATGTC TCACCTCAGG GGCCAGGGTA CTCTCTCACT TAACCACCCT GGTCAAGTGA 720

GCATCTTCTG GCTGGCTGTC TCCCCTCACT ATGAAAACCC CAAACTTCTA CCAATAACGG 780

GATTTGGGTT CTGTTATGAT AACTGTGACA CACACACACA CTCACACTCT GATAAAAGAG 840

AACTCTGATA AAAGAGATGG AAGACACTAA AAAAAAAAAA AAAAAA 886

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 102 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(F) TISSUE TYPE: mouse embryo

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Gly Arg Pro Val Val Pro Trp Ile Asp Val Tyr Ala Arg Ala Thr Cys
1 5 10 15

Gln Pro Arg Glu Val Val Val Pro Leu Ser Met Glu Leu Met Gly Asn
20 25 30

```
Gly Cys Cys Pro Asp Asp Gly Leu Glu Cys Val Pro Thr Gly Gln His
    50                  55                  60

Gln Val Arg Met Gln Ile Leu Met Ile Gln Tyr Pro Ser Ser Gln Leu
65                  70                  75                  80

Gly Glu Met Ser Leu Glu Glu His Ser Gln Cys Glu Cys Arg Pro Lys
                85                  90                  95

Lys Lys Arg Arg Val Leu
            100
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 55 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(F) TISSUE TYPE: mouse embryo

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Lys Pro Asp Ser Pro Arg Ile Leu Cys Pro Pro Cys Thr Gln Arg Arg
1               5                   10                  15

Gln Arg Pro Asp Pro Arg Thr Cys Arg Cys Arg Cys Arg Arg Arg Arg
            20                  25                  30

Phe Leu His Cys Gln Gly Arg Gly Leu Glu Leu Asn Pro Asp Thr Cys
        35                  40                  45

Arg Cys Arg Lys Pro Arg Lys
    50                  55
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 565 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(F) TISSUE TYPE: adult mouse heart

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GAGCCCCCTG CTCCGTCGCC TGCTGCTTGT TGCACTGCTG CAGCTGGCTC GCACCCAGGC 60

```
TTATGCACGT GCCACATGCC AGCCCAGGGA GGTGGTGGTG CCTCTGAGCA TGGAACTCAT      180

GGGCAATGTG GTCAAACAAC TAGTGCCCAG CTGTGTGACT GTGCAGCGCT GTGGTGGCTG      240

CTGCCCTGAC GATGGCCTGG AATGTGTGCC CACTGGGCAA CACCAAGTCC GAATGCAGAT      300

CCTCATGATC CAGTACCCGA GCAGTCAGCT GGGGGAGATG TCCCTGGAAG AACACAGCCA      360

ATGTGAATGC AGACCAAAAA AAAAGGAGAG TGCTGTGAAG CCAGACAGCC CCAGGATCCT      420

CTGCCCGCCT TGCACCCAGC GCCGTCAACG CCCTGACCCC CGGACCTGCC GCTGCCGCTG      480

CAGACGCCGC CGCTTCCTCC ATTGCCAAGG GCGGGGCTTA GAGCTCAACC CAGACACCTG      540

TAGGTGCCGG AAGCCGCGAA AGTGA                                            565
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 188 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(F) TISSUE TYPE: adult mouse heart

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Met Ser Pro Leu Leu Arg Arg Leu Leu Leu Val Ala Leu Leu Gln Leu
1               5                  10                  15

Ala Arg Thr Gln Ala Pro Val Ser Gln Phe Asp Gly Pro Ser His Gln
            20                  25                  30

Lys Lys Val Val Pro Trp Ile Asp Val Tyr Ala Arg Ala Thr Cys Gln
        35                  40                  45

Pro Arg Glu Val Val Val Pro Leu Ser Met Glu Leu Met Gly Asn Val
    50                  55                  60

Val Lys Gln Leu Val Pro Ser Cys Val Thr Val Gln Arg Cys Gly Gly
65                  70                  75                  80

Cys Cys Pro Asp Asp Gly Leu Glu Cys Val Pro Thr Gly Gln His Gln
                85                  90                  95

Val Arg Met Gln Ile Leu Met Ile Gln Tyr Pro Ser Ser Gln Leu Gly
            100                 105                 110

Glu Met Ser Leu Glu Glu His Ser Gln Cys Glu Cys Arg Pro Lys Lys
        115                 120                 125

Lys Glu Ser Ala Val Lys Pro Asp Ser Pro Arg Ile Leu Cys Pro Pro
    130                 135                 140

Cys Thr Gln Arg Arg Gln Arg Pro Asp Pro Arg Thr Cys Arg Cys Arg
145
```

Cys Arg Arg Arg Arg Phe Leu His Cys Gln Gly Arg Gly Leu Glu Leu
165 170 175

Asn Pro Asp Thr Cys Arg Cys Arg Lys Pro Arg Lys
180 185

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 591 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(F) TISSUE TYPE: adult mouse heart

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ACCATGAGCC CCCTGCTCCG TCGCCTGCTG CTTGTTGCAC TGCTGCAGCT GGCTCGCACC 60

CAGGCCCCTG TGTCCCAGTT TGATGGCCCC AGCCACCAGA AGAAAGTGGT GCCATGGATA 120

GACGTTTATG CACGTGCCAC ATGCCAGCCC AGGGAGGTGG TGGTGCCTCT GAGCATGGAA 180

CTCATGGGCA ATGTGGTCAA ACAACTAGTG CCCAGCTGTG TGACTGTGCA GCGCTGTGGT 240

GGCTGCTGCC CTGACGATGG CCTGGAATGT GTGCCCACTG GGCAACACCA AGTCCGAATG 300

CAGGTACCAG GGCCTATGGG TCAGATCCTC ATGATCCAGT ACCCGAGCAG TCAGCTGGGG 360

GAGATGTCCC TGGAAGAACA CAGCCAATGT GAATGCAGAC CAAAAAAAAA GGAGAGTGCT 420

GTGAAGCCAG ACAGCCCCAG GATCCTCTGC CCGCCTTGCA CCCAGCGCCG TCAACGCCCT 480

GACCCCCGGA CCTGCCGCTG CCGCTGCAGA CGCCGCCGCT TCCTCCATTG CCAAGGGCGG 540

GGCTTAGAGC TCAACCCAGA CACCTGTAGG TGCCGGAAGC CGCGAAAGTG A 591

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 195 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(F) TISSUE TYPE: adult mouse heart

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Ala Arg Thr Gln Ala Pro Val Ser Gln Phe Asp Gly Pro Ser His Gln
20 25 30

Lys Lys Val Val Pro Trp Ile Asp Val Tyr Ala Arg Ala Thr Cys Gln
35 40 45

Pro Arg Glu Val Val Val Pro Leu Ser Met Glu Leu Met Gly Asn Val
50 55 60

Val Lys Gln Leu Val Pro Ser Cys Val Thr Val Gln Arg Cys Gly Gly
65 70 75 80

Cys Cys Pro Asp Asp Gly Leu Glu Cys Val Pro Thr Gly Gln His Gln
85 90 95

Val Arg Met Gln Val Pro Gly Pro Met Gly Gln Ile Leu Met Ile Gln
100 105 110

Tyr Pro Ser Ser Gln Leu Gly Glu Met Ser Leu Glu Glu His Ser Gln
115 120 125

Cys Glu Cys Arg Pro Lys Lys Lys Glu Ser Ala Val Lys Pro Asp Ser
130 135 140

Pro Arg Ile Leu Cys Pro Pro Cys Thr Gln Arg Arg Gln Arg Pro Asp
145 150 155 160

Pro Arg Thr Cys Arg Cys Arg Cys Arg Arg Arg Arg Phe Leu His Cys
165 170 175

Gln Gly Arg Gly Leu Glu Leu Asn Pro Asp Thr Cys Arg Cys Arg Lys
180 185 190

Pro Arg Lys
195

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 405 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ACCATGAGCC CCCTGCTCCG TCGCCTGCTG CTTGTTGCAC TGCTGCAGCT GGCTCGCACC 60

CAGGCCCCTG TGTCCCAGTT TGATGGCCCC AGCCACCAGA AGAAAGTGGT GCCATGGATA 120

GACGTTTATG CACGTGCCAC ATGCCAGCCC AGGGAGGTGG TGGTGCCTCT GAGCATGGAA 180

CTCATGGGCA ATGTGGTCAA ACAACTAGTG CCCAGCTGTG TGACTGTGCA GCGCTGTGGT 240

GGCTGCTGCC CTGACGATGG CCTGGAATGT GTGCCCACTG GGCAACACCA AGTCCGAATG 300

CAGATCCTCA TGATCCAGTA CCCGAGCAGT CAGCTGGGGG AGATGTCCCT GGAAGAACAC 360

AGCCAATGTG AATGCAGACC AAAAAAAAAA AGGAGAGTGC TGTGA 405

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 133 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Met Ser Pro Leu Leu Arg Arg Leu Leu Leu Val Ala Leu Leu Gln Leu
1 5 10 15

Ala Arg Thr Gln Ala Pro Val Ser Gln Phe Asp Gly Pro Ser His Gln
20 25 30

Lys Lys Val Val Pro Trp Ile Asp Val Tyr Ala Arg Ala Thr Cys Gln
35 40 45

Pro Arg Glu Val Val Val Pro Leu Ser Met Glu Leu Met Gly Asn Val
50 55 60

Val Lys Gln Leu Val Pro Ser Cys Val Thr Val Gln Arg Cys Gly Gly
65 70 75 80

Cys Cys Pro Asp Asp Gly Leu Glu Cys Val Pro Thr Gly Gln His Gln
85 90 95

Val Arg Met Gln Ile Leu Met Ile Gln Tyr Pro Ser Ser Gln Leu Gly
100 105 110

Glu Met Ser Leu Glu Glu His Ser Gln Cys Glu Cys Arg Pro Lys Lys
115 120 125

Lys Arg Arg Val Leu
130

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 570 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(F) TISSUE TYPE: human fibrosarcoma

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ACCATGAGCC CTCTGCTCCG CCGCCTGCTG CTCGCCGCAC TCCTGCAGCT GGCCCCCGCC 60

CAGGCCCCTG TCTCCCAGCC TGATGCCCCT GGCCACCAGA GGAAAGTGGT GTCATGGATA 120

GATGTGTATA CTCGCGCTAC CTGCCAGCCC CGGGAGGTGG TGGTGCCCTT GACTGTGGAG 180

GGCTGCTGCC CTGACGATGG CCTGGAGTGT GTGCCCACTG GGCAGCACCA AGTCCGGATG 300

CAGATCCTCA TGATCCGGTA CCCGAGCAGT CAGCTGGGGG AGATGTCCCT GGAAGAACAC 360

AGCCAGTGTG AATGCAGACC TAAAAAAAAG GACAGTGCTG TGAAGCCAGA CAGCCCCAGG 420

CCCCTCTGCC CACGCTGCAC CCAGCACCAC CAGCGCCCTG ACCCCCGGAC CTGCCGCTGC 480

CGCTGCCGAC GCCGCAGCTT CCTCCGTTGC CAAGGGCGGG GCTTAGAGCT CAACCCAGAC 540

ACCTGCAGGT GCCGGAAGCT GCGAAGGTGA 570

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 188 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(F) TISSUE TYPE: human fibrosarcoma

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Met Ser Pro Leu Leu Arg Arg Leu Leu Leu Ala Ala Leu Leu Gln Leu
1 5 10 15

Ala Pro Ala Gln Ala Pro Val Ser Gln Pro Asp Ala Pro Gly His Gln
20 25 30

Arg Lys Val Val Ser Trp Ile Asp Val Tyr Thr Arg Ala Thr Cys Gln
35 40 45

Pro Arg Glu Val Val Val Pro Leu Thr Val Glu Leu Met Gly Thr Val
50 55 60

Ala Lys Gln Leu Val Pro Ser Cys Val Thr Val Gln Arg Cys Gly Gly
65 70 75 80

Cys Cys Pro Asp Asp Gly Leu Glu Cys Val Pro Thr Gly Gln His Gln
85 90 95

Val Arg Met Gln Ile Leu Met Ile Arg Tyr Pro Ser Ser Gln Leu Gly
100 105 110

Glu Met Ser Leu Glu Glu His Ser Gln Cys Glu Cys Arg Pro Lys Lys
115 120 125

Lys Asp Ser Ala Val Lys Pro Asp Ser Pro Arg Pro Leu Cys Pro Arg
130 135 140

Cys Thr Gln His His Gln Arg Pro Asp Pro Arg Thr Cys Arg Cys Arg
145 150 155 160

Cys Arg Arg Arg Ser Phe Leu Arg Cys Gln Gly Arg Gly Leu Glu Leu
165 170 175

Asp [illegible]

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 624 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(F) TISSUE TYPE: mouse

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ATGAGCCCCC TGCTCCGTCG CCTGCTGCTT GTTGCACTGC TGCAGCTGGC TCGCACCCAG 60

GCCCCTGTGT CCCAGTTTGA TGGCCCCAGC CACCAGAAGA AAGTGGTGCC ATGGATAGAC 120

GTTTATGCAC GTGCCACATG CCACCCCAGG GAGGTGGTGG TGCCTCTGAG CATGGAACTC 180

ATGGGCAATG TGGTCAAACA ACTAGTGCCC AGCTGTGTGA CTGTGCAGCG CTGTGGTGGC 240

TGCTGCCCTG ACGATGGCCT GGAATGTGTG CCCACTGGGC AACACCAAGT CCGAATGCAG 300

ATCCTCATGA TCCAGTACCC GAGCAGTCAG CTGGGGGAGA TGTCCCTGGA AGAACACAGC 360

CAATGTGAAT GCAGACCAAA AAAAAAGGAG AGTGCTGTGA AGCCAGACAG GGTTGCCATA 420

CCCCACCACC GTCCCCAGCC CCGCTCTGTT CCGGGCTGGG ACTCTACCCC GGGAGCATCC 480

TCCCCAGCTG ACATCATCCA TCCCACTCCA GCCCCAGGAT CCTCTGCCCG CCTTGCACCC 540

AGCGCCGTCA ACGCCCTGAC CCCCGGACCT GCCGCTGCCG CTGCAGACGC CGCCGCTTCC 600

TCCATTGCCA AGGGCGGGGC TTAG 624

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 207 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(F) TISSUE TYPE: mouse

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Met Ser Pro Leu Leu Arg Arg Leu Leu Leu Val Ala Leu Leu Gln Leu
1 5 10 15

Ala Arg Thr Gln Ala Pro Val Ala Gln Phe Asp Gly Pro Ser His Gln
20 25 30

```
Pro Arg Glu Val Val Val Pro Leu Ser Met Glu Leu Met Gly Asn Val
    50                  55                  60

Val Lys Gln Leu Val Pro Ser Cys Val Thr Val Gln Arg Cys Gly Gly
65                  70                  75                  80

Cys Cys Pro Asp Asp Gly Leu Glu Cys Val Pro Thr Gly Gln His Gln
                85                  90                  95

Val Arg Met Gln Ile Leu Met Ile Gln Tyr Pro Ser Ser Gln Leu Gly
            100                 105                 110

Glu Met Ser Leu Glu Glu His Ser Gln Cys Glu Cys Arg Pro Lys Lys
        115                 120                 125

Lys Glu Ser Ala Val Lys Pro Asp Arg Val Ala Ile Pro His His Arg
    130                 135                 140

Pro Gln Pro Arg Ser Val Pro Gly Trp Asp Ser Thr Pro Gly Ala Ser
145                 150                 155                 160

Ser Pro Ala Asp Ile Ile His Pro Thr Pro Ala Pro Gly Ser Ser Ala
                165                 170                 175

Arg Leu Ala Pro Ser Ala Val Asn Ala Leu Thr Pro Gly Pro Ala Ala
            180                 185                 190

Ala Ala Ala Asp Ala Ala Ala Ser Ser Ile Ala Lys Gly Gly Ala
        195                 200                 205
```

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 624 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(F) TISSUE TYPE: human

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
ATGAGCCCTC TGCTCCGCCG CCTGCTGCTC GCCGCACTCC TGCAGCTGGC CCCCGCCCAG      60

GCCCCTGTCT CCCAGCCTGA TGCCCCTGGC CACCAGAGGA AAGTGGTGTC ATGGATAGAT     120

GTGTATACTC GCGCTACCTG CCAGCCCCGG GAGGTGGTGG TGCCCTTGAC TGTGGAGCTC     180

ATGGGCACCG TGGCCAAACA GCTGGTGCCC AGCTGCGTGA CTGTGCAGCG CTGTGGTGGC     240

TGCTGCCCTG ACGATGGCCT GGAGTGTGTG CCCACTGGGC AGCACCAAGT CCGGATGCAG     300

ATCCTCATGA TCCGGTACCC GAGCAGTCAG CTGGGGGAGA TGTCCCTGGA AGAACACAGC     360

CAGTGTGAAT GCAGACCTAA AAAAAAGGAC AGTGCTGTGA AGCCAGACAG GGCTGCCACT     420

CCCCACCACC GTCCCCAGCC CCGTTCTGTT CCGGGCTGGG ACTCTGCCCC CGGAGCACCC     480
```

AGCACCACCA GCGCCCTGAC CCCCGGACCT GCCGCCGCCG CTGCCGACGC CGCAGCTTCC 600

TCCGTTGCCA AGGGCGGGGC TTAG 624

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 207 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(F) TISSUE TYPE: human

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
Met Ser Pro Leu Leu Arg Arg Leu Leu Leu Ala Ala Leu Leu Gln Leu
1               5                  10                  15

Ala Pro Ala Gln Ala Pro Val Ser Gln Pro Asp Ala Pro Gly His Gln
            20                  25                  30

Arg Lys Val Val Ser Trp Ile Asp Val Tyr Thr Arg Ala Thr Cys Gln
        35                  40                  45

Pro Arg Glu Val Val Val Pro Leu Thr Val Glu Leu Met Gly Thr Val
    50                  55                  60

Ala Lys Gln Leu Val Pro Ser Cys Val Thr Val Gln Arg Cys Gly Gly
65                  70                  75                  80

Cys Cys Pro Asp Asp Gly Leu Glu Cys Val Pro Thr Gly Gln His Gln
                85                  90                  95

Val Arg Met Gln Ile Leu Met Ile Arg Tyr Pro Ser Ser Gln Leu Gly
            100                 105                 110

Glu Met Ser Leu Glu Glu His Ser Gln Cys Glu Cys Arg Pro Lys Lys
        115                 120                 125

Lys Asp Ser Ala Val Lys Pro Asp Arg Ala Ala Thr Pro His His Arg
    130                 135                 140

Pro Gln Pro Arg Ser Val Pro Gly Trp Asp Ser Ala Pro Gly Ala Pro
145                 150                 155                 160

Ser Pro Ala Asp Ile Thr His Pro Thr Pro Ala Pro Gly Pro Ser Ala
                165                 170                 175

His Ala Ala Pro Ser Thr Thr Ser Ala Leu Thr Pro Gly Pro Ala Ala
            180                 185                 190

Ala Ala Ala Asp Ala Ala Ala Ser Ser Val Ala Lys Gly Gly Ala
        195                 200                 205
```

(2) INFORMATION FOR SEQ ID NO:16:

(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Pro Xaa Cys Val Xaa Xaa Xaa Arg Cys Xaa Gly Cys Cys
1 5 10

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1550 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CTCGAGATCT GTTTGTTGTC TTGGAACAAT ACGGTTTAGA GGTGACTGGC GGGTGACGAG 60

AACATATGCG AGTTCACCTA AGAGAAAAGC TGAATGAGGC AATGCCTCTT CCTGACCATA 120

TCTCTTACTC AGATAACTAT AGAATTTATT GTCCAGTAAA GGGTATATTA AAAAATCATA 180

TTAAAAGTCA TACAGTGAAG TTGTCCAGGG AAATCAAGAC TTAACAGTCT CACTCTGACA 240

ATAATGAACA GGGGGATTCC CTCAAGATAG ACTAGGACAT GACCCCACAC TGGCAGGTAG 300

TAGTACCAGA AAAGAACGCA TGGAAAATCT TTACCTTATG CTTGAGGTAG GGACCAGGCT 360

AAAGTGAAGG CCAGACCTAA AATTCTATCT AAAATAAATC CACAATCGAA GAAAATATGT 420

GGTGTACAGG TATAGAATGT CTTTACTGGA TCATTGAAAT AGTAAGATAA ATTCAACTTT 480

TTACATTGTT TTCTTTTCCT CCAGTTAGGG CTTGAGACCT TCGTCTCTGG AGAGTGACTG 540

TCAATTGGAG CCCTGCTTTC TGGGTTTCTG GCCAGGGGGG TTGTGGATGC TTAACATGTG 600

CCTTTCACAG GACACTTCCT TACCCCAGCA GTGGCCANGT GTGCATCCCA CGACCAGGCC 660

TCCCTCTCAC GGAACATCTG TTGAGACTAG GAGATGCCTG GTGACTGTTG CCTGACCTGT 720

GTCCTGTGTA TTTCTGACAA GAGCCACTCT CAAAGACCCT GGCCAGGAGG AGAGTTAGGT 780

TCCAGTGTAG GTCAGCTCAG ACAGATGGAG GCCACAGAAN CAAACATGGG AAATCACAGA 840

AGTAGGTTTA TTACTCACAG ATCCCTATCC CAACCACCCA GGTGCCCTCT CCTCCAGGGC 900

CAACAGAGGC ATCCTTCAGC AGGAGCGACA ACGGCTAGGG CAGCGGCAAG CCGCCACCAT 960

CCGAGCCAAC CCAGGCCCCG AGATCGTGCC CCGGGGCGCC GGCCCCTGAG GGGCTCACCT 1020

GGATGGGGCC TGCATGCGTT CCCGCTTTGC TTCCTTCCCT GGACGGCCCG CTCCCCCGAA 1080

ACGCGCCGCC AATAAAGTGA TTCGCAGAGC TCGTGTGCGG CTCCCTCCTT AAGGCCCGAC 1140

ACTGGGGAGC CCAGCCTCCT GGGCGGTGCG TCCCCTTCCC CCTGCCGCGG CGGGAGGCGG 1260

GAGGGGGTGT GTGGAGGAGG CGGGCCCCGC CGACGGCCTC GCCCCCCCAC CCCGCCGCCC 1320

CGCCCCCGCC CCACGGGCCC GGTGGGGAGC GCGTGTCTGG GTCACATGAG CCGCCTGCCC 1380

GCCAGCCCGG GCCCAGCCCC CCGCCGCCCC CGCCGTCCCC GCCGCCGCTG CCCGCCGCCA 1440

CCGGCCGCCC GCCCGCCCGG CTCCTCCGGC CGCCTTCGCT GCGCTGCNTG CGCTGCCTGC 1500

ACCCAGGGCT CGGGAGGGGG CCGCGGAGGA GCCGCCCCCC GCGCCCGGCC 1550

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CACCATGAGC CCTCTGCTCC 20

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GCCATGTGTC ACCTTCGCAG 20

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GGGCATCAGG CTGGGAGACA G 21

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Cys Pro Val Ser Gln Phe Asp Gly Pro Ser His Gln Lys Lys Val Val
1 5 10 15

Pro Cys

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Ser Gln Pro Asp Ala Pro Gly His Gln Arg Lys Val Val Ser Trp Ile
1 5 10 15

Asp Val Tyr Thr Arg Ala Thr
20

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CACAGCCAAT GTGAATGCA 19

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GCTCTAAGCC CCGCCCTTGG CAATGGAGGA A 31

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

ACGTAGATCT TCACTTTCGC GGCTTCCG 28

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CTCTGTTCCG GGCTGGGACT CTA 23

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

TCAGGGCGTT GACGGCGCTG GGTGCAA 27

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CCTGACGATG GCCTGGAGTG T 21

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs

(D) TOPOLOGY linear

(ii) MOLECULE TYPE cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

TGTCCCTGGA AGAACACAGC C 21

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

CGGGAAATCG TGCGTGACAT 20

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GGAGTTGAAG GTAGTTTCGT G 21

What is claimed is:

1. An isolated DNA which codes for a protein which comprises a characteristic amino acid sequence

Pro-Xaa-Cys-Val-Xaa-Xaa-Xaa-Arg-Cys-Xaa-Gly-Cys-Cys

(SEQ ID NO:16) and has the property of promoting proliferation of endothelial cells or mesodermal cells, said DNA being selected from the group consisting of the DNA of Figures 1 and 2 (SEQ ID NO:1), the DNA of Figure 3 (SEQ ID NO:4), the DNA of Figure 5 (SEQ ID NO:6), the DNA of Figure 7 (SEQ ID NO:8), the DNA of Figure 10 (SEQ ID NO:10), the DNA of Figure 12 (SEQ ID NO:12), the DNA of Figure 14 (SEQ ID NO:14), and DNA which hybridizes under stringent conditions with at least one of the foregoing DNA sequences.

2. A DNA according to claim 1, wherein said DNA is a cDNA.

3. A DNA according to claim 1, comprising a cDNA corresponding to the DNA of Figures 1 and 2 (SEQ ID NO:1).

4. A DNA according to claim 1, wherein said DNA sequence is a mammalian DNA.

5. A DNA according to claim 4, wherein said DNA is a murine DNA.

6. A DNA according to claim 4, wherein said DNA is a human DNA.

7. A DNA according to claim 1, wherein said DNA codes for a protein which promotes proliferation of vascular endothelial cells.

8. A DNA according to claim 1, comprising a cDNA

9. A DNA according to claim 1, comprising a cDNA corresponding to the DNA of Figure 5 (SEQ ID NO:6).

10. A DNA according to claim 1, comprising a cDNA corresponding to the DNA of Figure 7 (SEQ ID NO:8).

11. A DNA according to claim 1, comprising a cDNA corresponding to the DNA of Figure 10 (SEQ ID NO:10).

12. A DNA according to claim 1, comprising a cDNA corresponding to the DNA of Figure 12 (SEQ ID NO:12).

13. A DNA according to claim 1, comprising a cDNA corresponding to the DNa of Figure 14 (SEQ ID NO:14).

14. A vector comprising a DNA according to claim 1.

15. A vector according to claim 14, wherein said vector is a eukaryotic vector.

16. A vector according to claim 14, wherein said vector is a prokaryotic vector.

17. A vector according to claim 14, wherein said vector is a plasmid.

18. A protein which exhibits a characteristic sequence

Pro-Xaa-Cys-Val-Xaa-Xaa-Xaa-Arg-Cys-Xaa-Gly-Cys-Cys

(SEQ ID NO:16) and has the property of promoting proliferation of endothelial cells or mesodermal cells, said protein comprising a sequence of amino acids substantially corresponding to an amino acid sequence selected from the group consisting of the amino acid sequence of Figure 1 (SEQ ID NO:2), the amino acid sequence of Figure 2 (SEQ ID NO:3), the amino acid sequence of Figure 4 (SEQ ID NO:5), the amino

sequence of Figure 8 (SEQ ID NO:9), the amino acid sequence of Figure 11 (SEQ ID NO:11), the amino acid sequence of Figure 13 (SEQ ID NO:13), and the amino acid sequence of Figure 15 (SEQ ID NO:15).

19. A protein according to claim 18, wherein said protein comprises a sequence of amino acids corresponding to the amino acid sequence of Figure 1 (SEQ ID NO:2).

20. A protein according to claim 18, wherein said protein comprises an amino acid sequence corresponding to the amino acid sequence of Figure 2 (SEQ ID NO:3).

21. A protein according to claim 18, wherein said protein comprises a sequence of amino acids corresponding to the amino acid sequence of Figure 4 (SEQ ID NO:5).

22. A protein according to claim 18, wherein said protein comprises a sequence of amino acids corresponding to the amino acid sequence of Figure 6 (SEQ ID NO:7).

23. A protein according to claim 18, wherein said protein comprises a sequence of amino acids corresponding to the amino acid sequence of Figure 8 (SEQ ID NO:9).

24. A protein according to claim 18, wherein said protein comprises a sequence of amino acids corresponding to the amino acid sequence of Figure 11 (SEQ ID NO:11).

25. A protein according to claim 18, wherein said protein comprises a sequence of amino acids corresponding to the amino acid sequence of Figure 13 (SEQ ID NO:13).

26. A protein according to claim 18, wherein said protein comprises a sequence of amino acids corresponding to

27. A protein according to claim 18, wherein said protein is a mammalian protein.

28. A protein according to claim 27, wherein said protein is a murine protein.

29. A protein according to claim 27, wherein said protein is a human protein.

30. A protein according to claim 18, wherein said protein promotes proliferation of vascular endothelial cells.

31. A protein produced by expression of a DNA selected from the group consisting of the DNA of Figures 1 and 2 (SEQ ID NO:1), the DNA of Figure 3 (SEQ ID NO:4), the DNA of Figure 5 (SEQ ID NO:6), the DNA of Figure 7 (SEQ ID NO:8), the DNA of Figure 10 (SEQ ID NO:10), the DNA of Figure 12 (SEQ ID NO:12), the DNA of Figure 14 (SEQ ID NO:14), and DNA which hybridizes under stringent conditions with at least one of the foregoing DNA sequences.

32. A pharmaceutical composition comprising an effective endothelial or mesodermal cell proliferation promoting amount of a protein according to claim 18, and at least one conventional pharmaceutical carrier or diluent.

33. An antibody which reacts with a protein according to claim 18.

34. An antibody according to claim 33, wherein said antibody is a monoclonal antibody.

35. A host cell transformed or transfected with a vector according to claim 14, such that said host cell expresses a protein having the property of promoting proliferation of endothelial or mesodermal cells.

36. A transfected host cell according to claim 35, wherein said host cell is a eukaroytic cell.

37. A transfected host cell according to claim 35, wherein said host cell is a COS cell.

38. A transformed host cell according to claim 35, wherein said host cell is a prokaryotic cell.

39. A transformed host cell according to claim 35, wherein said host cell is a 293EBNA cell.

40. A transformed host cell according to claim 35, wherein said host cell is an insect cell.

41. A diagnostic means for quantitatively detecting VEGF-B in a test sample, said means comprising an antibody according to claim 33, which reacts with VEGF-B in order to detect the amount of VEGF-B in the sample.

42. A diagnostic means according to claim 41, wherein said antibody is a labelled antibody.

43. A diagnostic means for detecting VEGF-B in a test sample, said means comprising at least one pair of primers complementary to a DNA sequence according to claim 1, for amplifying a test VEGF-B gene by polymerase chain reaction in order to facilitate a sequence comparison of the test VEGF-B gene with the DNA sequence according to claim 1.

44. A pharmaceutical composition comprising an effective VEGF-B-binding amount of antibodies according to claim 33.

45. A pharmaceutical composition comprising an effective endothelial or mesodermal cell proliferation promoting amount of a protein according to claim 18, and VEGF.

46. A pharmaceutical composition according to claim 45, further comprising an amount of heparin effective to assure that a cell proliferation promoting amount of said protein is present free from association with a cell.

47. A pharmaceutical composition according to claim 32, further comprising an amount of heparin effective to assure that a cell proliferation promoting amount of said protein is present free from association with a cell.

48. A pharmaceutical composition comprising an effective angiogenesis stimulating amount of a protein according to claim 18, and heparin.

49. A protein dimer comprising a protein according to claim 18.

50. A protein dimer according to claim 49, wherein said protein dimer is a homodimer of said protein.

51. A protein dimer according to claim 49, wherein said protein dimer is a heterodimer of said protein and VEGF.

52. A protein dimer according to claim 49, wherein said protein dimer is a disulfide-linked dimer.

53. A method of promoting release of at least one protein selected from the group consisting of proteins according to claim 18, and VEGF from a cell which expresses said at least one protein, said method comprising exposing the cell to heparin.

54. A vector comprising an anti-sense nucleotide sequence complementary to at least part of a DNA sequence according to claim 1.

55. A method of retarding expression of VEGF-B from a cell which expresses VEGF-B, said method comprising transfecting said cell with a vector according to claim 54.

56. A method according to claim 55, wherein said cell is a tumor cell.

57. An isolated DNA comprising a nucleotide sequence corresponding to the sequence of Figure 25 (SEQ ID NO:17) or a DNA which hybridizes under stringent conditions to said nucleotide sequence.

AMENDED CLAIMS

[received by the International Bureau on 14 August 1996 (14.08.96); original claims 1-14, 18-31, 43, 44, 49-52 and 57 amended; new claims 58-63 added; remaining claims unchanged (8 pages)]

1. An isolated nucleic acid which codes for a protein which comprises a characteristic amino acid sequence

Pro-Xaa-Cys-Val-Xaa-Xaa-Xaa-Arg-Cys-Xaa-Gly-Cys-Cys

(SEQ ID NO:16) and has the property of promoting proliferation of endothelial cells or mesodermal cells, said nucleic acid being selected from the group consisting of the DNA of Figures 1 and 2 (SEQ ID NO:1), the DNA of Figure 3 (SEQ ID NO:4), the DNA of Figure 5 (SEQ ID NO:6), the DNA of Figure 7 (SEQ ID NO:8), the DNA of Figure 10 (SEQ ID NO:10), the DNA of Figure 12 (SEQ ID NO:12), the DNA of Figure 14 (SEQ ID NO:14), and nucleic acid which hybridizes under stringent conditions with at least one of the foregoing DNA sequences.

2. A nucleic acid according to claim 1, wherein said nucleic acid is a cDNA.

3. A nucleic acid according to claim 1, comprising a cDNA corresponding to the DNA of Figures 1 and 2 (SEQ ID NO:1).

4. A nucleic acid according to claim 1, wherein said nucleic acid sequence is a mammalian DNA.

5. A nucleic acid according to claim 4, wherein said nucleic acid is a murine DNA.

6. A nucleic acid according to claim 4, wherein said nucleic acid is a human DNA.

7. A nucleic acid according to claim 1, wherein said nucleic acid codes for a protein which promotes proliferation of vascular endothelial cells.

9. A nucleic acid according to claim 1, comprising a cDNA corresponding to the DNA of Figure 5 (SEQ ID NO:6).

10. A nucleic acid according to claim 1, comprising a cDNA corresponding to the DNA of Figure 7 (SEQ ID NO:8).

11. A nucleic acid according to claim 1, comprising a cDNA corresponding to the DNA of Figure 10 (SEQ ID NO:10).

12. A nucleic acid according to claim 1, comprising a cDNA corresponding to the DNA of Figure 12 (SEQ ID NO:12).

13. A nucleic acid according to claim 1, comprising a cDNA corresponding to the DNa of Figure 14 (SEQ ID NO:14).

14. A vector comprising a nucleic acid according to claim 1, operably linked with a promoter sequence.

15. A vector according to claim 14, wherein said vector is a eukaryotic vector.

16. A vector according to claim 14, wherein said vector is a prokaryotic vector.

17. A vector according to claim 14, wherein said vector is a plasmid.

18. An isolated protein which exhibits a characteristic sequence

Pro-Xaa-Cys-Val-Xaa-Xaa-Xaa-Arg-Cys-Xaa-Gly-Cys-Cys

(SEQ ID NO:16) and has the property of promoting proliferation of endothelial cells or mesodermal cells, said protein comprising a sequence of amino acids substantially corresponding to an amino acid sequence selected from the group consisting of the amino acid sequence of Figure 1 (SEQ ID NO:2), the amino acid sequence of Figure 2 (SEQ ID NO:3), the amino acid sequence of [illegible]

sequence of Figure 8 (SEQ ID NO:9), the amino acid sequence of Figure 11 (SEQ ID NO:11), the amino acid sequence of Figure 13 (SEQ ID NO:13), and the amino acid sequence of Figure 15 (SEQ ID NO:15).

19. An isolated protein according to claim 18, wherein said protein comprises a sequence of amino acids corresponding to the amino acid sequence of Figure 1 (SEQ ID NO:2).

20. An isolated protein according to claim 18, wherein said protein comprises an amino acid sequence corresponding to the amino acid sequence of Figure 2 (SEQ ID NO:3).

21. An isolated protein according to claim 18, wherein said protein comprises a sequence of amino acids corresponding to the amino acid sequence of Figure 4 (SEQ ID NO:5).

22. An isolated protein according to claim 18, wherein said protein comprises a sequence of amino acids corresponding to the amino acid sequence of Figure 6 (SEQ ID NO:7).

23. An isolated protein according to claim 18, wherein said protein comprises a sequence of amino acids corresponding to the amino acid sequence of Figure 8 (SEQ ID NO:9).

24. An isolated protein according to claim 18, wherein said protein comprises a sequence of amino acids corresponding to the amino acid sequence of Figure 11 (SEQ ID NO:11).

25. An isolated protein according to claim 18, wherein said protein comprises a sequence of amino acids corresponding to

AMENDED SHEET (ARTICLE 19)

26. An isolated protein according to claim 18, wherein said protein comprises a sequence of amino acids corresponding to the amino acid sequence of Figure 15 (SEQ ID NO:15).

27. An isolated protein according to claim 18, wherein said protein is a mammalian protein.

28. An isolated protein according to claim 27, wherein said protein is a murine protein.

29. An isolated protein according to claim 27, wherein said protein is a human protein.

30. An isolated protein according to claim 18, wherein said protein promotes proliferation of vascular endothelial cells.

31. An isolated protein produced by expression of a DNA selected from the group consisting of the DNA of Figures 1 and 2 (SEQ ID NO:1), the DNA of Figure 3 (SEQ ID NO:4), the DNA of Figure 5 (SEQ ID NO:6), the DNA of Figure 7 (SEQ ID NO:8), the DNA of Figure 10 (SEQ ID NO:10), the DNA of Figure 12 (SEQ ID NO:12), the DNA of Figure 14 (SEQ ID NO:14), and DNA which hybridizes under stringent conditions with at least one of the foregoing DNA sequences.

32. A pharmaceutical composition comprising an effective endothelial or mesodermal cell proliferation promoting amount of a protein according to claim 18, and at least one conventional pharmaceutical carrier or diluent.

33. An antibody which reacts with a protein according to claim 18.

34. An antibody according to claim 33, wherein said

AMENDED SHEET (ARTICLE 19)

35. A host cell transformed or transfected with a vector according to claim 14, such that said host cell expresses a protein having the property of promoting proliferation of endothelial or mesodermal cells.

36. A transfected host cell according to claim 35, wherein said host cell is a eukaroytic cell.

37. A transfected host cell according to claim 35, wherein said host cell is a COS cell.

38. A transformed host cell according to claim 35, wherein said host cell is a prokaryotic cell.

39. A transformed host cell according to claim 35, wherein said host cell is a 293EBNA cell.

40. A transformed host cell according to claim 35, wherein said host cell is an insect cell.

41. A diagnostic means for quantitatively detecting VEGF-B in a test sample, said means comprising an antibody according to claim 33, which reacts with VEGF-B in order to detect the amount of VEGF-B in the sample.

42. A diagnostic means according to claim 41, wherein said antibody is a labelled antibody.

43. A diagnostic means for detecting VEGF-B in a test sample, said means comprising at least one pair of primers complementary to a nucleic acid sequence according to claim 1, for amplifying a test VEGF-B gene by polymerase chain reaction in order to facilitate a sequence comparison of the test VEGF-B gene with the nucleic acid sequence according to claim 1.

44. A pharmaceutical composition comprising an effective

45. A pharmaceutical composition comprising an effective endothelial or mesodermal cell proliferation promoting amount of a protein according to claim 18, and VEGF.

46. A pharmaceutical composition according to claim 45, further comprising an amount of heparin effective to assure that a cell proliferation promoting amount of said protein is present free from association with a cell.

47. A pharmaceutical composition according to claim 32, further comprising an amount of heparin effective to assure that a cell proliferation promoting amount of said protein is present free from association with a cell.

48. A pharmaceutical composition comprising an effective angiogenesis stimulating amount of a protein according to claim 18, and heparin.

49. An isolated protein dimer comprising a protein according to claim 18.

50. An isolated protein dimer according to claim 49, wherein said protein dimer is a homodimer of said protein.

51. An isolated protein dimer according to claim 49, wherein said protein dimer is a heterodimer of said protein and VEGF.

52. An isolated protein dimer according to claim 49, wherein said protein dimer is a disulfide-linked dimer.

53. A method of promoting release of at least one protein selected from the group consisting of proteins according to claim 18, and VEGF from a cell which expresses said at least one protein, said method comprising expos-

54. A vector comprising an anti-sense nucleotide sequence complementary to at least part of a nucleic acid sequence according to claim 1.

55. A method of retarding expression of VEGF-B from a cell which expresses VEGF-B, said method comprising transfecting said cell with a vector according to claim 54.

56. A method according to claim 55, wherein said cell is a tumor cell.

57. An isolated nucleic acid comprising a nucleotide sequence corresponding to the sequence of Figure 25 (SEQ ID NO:17) or a nucleic acid which hybridizes under stringent conditions to said nucleotide sequence.

58. A host cell transformed or transfected with vector comprising a nucleic acid sequence according to claim 1, operatively linked to a suitable promoter, such that said host cell expresses a VEGF-B protein.

59. A host cell according to claim 58, wherein said VEGF-B protein comprises a sequence of amino acids substantially corresponding to an amino acid sequence selected from the group consisting of the amino acid sequence of Figure 1 (SEQ ID NO:2), the amino acid sequence of Figure 2 (SEQ ID NO:3), the amino acid sequence of Figure 4 (SEQ ID NO:5), the amino acid sequence of Figure 6 (SEQ ID NO:7), the amino acid sequence of Figure 8 (SEQ ID NO:9), the amino acid sequence of Figure 11 (SEQ ID NO:11), the amino acid sequence of Figure 13 (SEQ ID NO:13), and the amino acid sequence of Figure 15 (SEQ ID NO:15).

60. A diagnostic means for detecting VEGF-B in a test sample, said means comprising at least one pair of primers complementary to a nucleic acid sequence

61. A method for obtaining at least one protein selected from the group consisting of proteins according to claim 18, and VEGF from a cell which expresses said at least one protein, said method comprising

- exposing the cell to heparin to induce release of said at least one protein, and
- collecting the released protein.

62. A method of making a vector suitable for expression of VEGF-B protein, said method comprising incorporating a nucleotide sequence according to claim 1, into a vector in operatively linked relation with a suitable promoter.

63. An isolated nucleic acid molecule which encodes a human VEGF-B molecule, wherein said isolated nucleic acid molecule hybridizes to a nucleic acid molecule encoding murine VEGF-B, at 42°C in 50% formamide, 5 x SSC pH 7.0 or 5 x SSPE buffer, 1% to 2% SDS, 5 to 10 x Denhardt's solution and 100 μg/ml of salmon sperm DNA.

```
                                                                    CG
Gly Arg Pro Val Val Pro Trp Ile Asp Val Tyr Ala Arg Ala Thr Cys Gln   17
GGA CGC CCA GTG GTG CCA TGG ATA GAC GTT TAT GCA CGT GCC ACA TGC CAG
Pro Arg Glu Val Val Val Pro Leu Ser Met Glu Leu Met Gly Asn Val Val   34
CCC AGG GAG GTG GTG GTG CCT CTG AGC ATG GAA CTC ATG GGC AAT GTG GTC
Lys Gln Leu Val Pro Ser Cys Val Thr Val Gln Arg Cys Gly Gly Cys Cys   51
AAA CAA CTA GTG CCC AGC TGT GTG ACT GTG CAG CGC TGT GGT GGC TGC TGC
Pro Asp Asp Gly Leu Glu Cys Val Pro Thr Gly Gln His Gln Val Arg Met   68
CCT GAC GAT GGC CTG GAA TGT GTG CCC ACT GGG CAA CAC CAA GTC CGA ATG
Gln Ile Leu Met Ile Gln Tyr Pro Ser Ser Gln Leu Gly Glu Met Ser Leu   85
CAG ATC CTC ATG ATC CAG TAC CCG AGC AGT CAG CTG GGG GAG ATG TCC CTG
Glu Glu His Ser Gln Cys Glu Cys Arg Pro Lys Lys Lys Arg Arg Val Leu  102
GAA GAA CAC AGC CAA TGT GAA TGC AGA CCA AAA AAA AAA AGG AGA GTG CTG
Stop
TGA AGCCAGACAGCCCCAGGATCCTCTGCCCGCCTTGCACCCAGCGCCGTCAACGCCCTGACCCCC
GGACCTGCCGCTGCCGCTGCAGACGCCGCCGCTTCCTCCATTGCCAAGGGCGGGGCTTAGAGCTCAA
CCCAGACACCTGTAGGTGCCGGAAGCCGCGAAAGTGACAAGCTGCTTTCCAGACTCCACGGGCCCGG
CTGCTTTTATGGCCCTGCTTCACAGGGACGAAGAGTGGAGCACAGGCAAACCTCCTCAGTCTGGGAG
GTCACTGCCCCAGGACCTGGACCTTTTAGAGAGCTCTCTCGCCATCTTTTATCTCCCAGAGCTGCCA
TCTAACAATTGTCAAGGAACCTCATGTCTCACCTCAGGGGCCAGGGTACTCTCTCACTTAACCACCC
TGGTCAAGTGAGCATCTTCTGGCTGGCTGTCTCCCCTCACTATGAAAACCCCAAACTTCTACCAATA
ACGGGATTTGGGTTCTGTTATGATAACTGTGACACACACACACACTCACACTCTGATAAAAGAGAAC
TCTGATAAAAGAGATGGAAGACACTAAAAAAAAAAAAAAAAAA
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ID NOS:1 & 2)

FIG. 1

```
                                                                  CGGGACGCC
CAGTGGTGCCATGGATAGACGTTTATGCACGTGCCACATGCCAGCCCAGGGAGGTGGTGGTGCCTCT
GAGCATGGAACTCATGGGCAATGTGGTCAAACAACTAGTGCCCAGCTGTGTGACTGTGCAGCGCTGT
GGTGGCTGCTGCCCTGACGATGGCCTGGAATGTGTGCCCACTGGGCAACACCAAGTCCGAATGCAGA
TCCTCATGATCCAGTACCCGAGCAGTCAGCTGGGGGAGATGTCCCTGGAAGAACACAGCCAATGTGA
                                                  Lys Pro Asp Ser Pro Arg     7
ATG CAG ACC AAA AAA AAA AAG GAG AGT GCT GTG AAG CCA GAC AGC CCC AGG
Ile Leu Cys Pro Pro Cys Thr Gln Arg Arg Gln Arg Pro Asp Pro Arg Thr    24
ATC CTC TGC CCG CCT TGC ACC CAG CGC CGT CAA CGC CCT GAC CCC CGG ACC
Cys Arg Cys Arg Cys Arg Arg Arg Arg Phe Leu His Cys Gln Gly Arg Gly    41
TGC CGC TGC CGC TGC AGA CGC CGC CGC TTC CTC CAT TGC CAA GGG CGG GGC
Leu Glu Leu Asn Pro Asp Thr Cys Arg Cys Arg Lys Pro Arg Lys Stop       56
TTA GAG CTC AAC CCA GAC ACC TGT AGG TGC CGG AAG CCG CGA AAG TGA CAA
GCTGCTTTCCAGACTCCACGGGCCCGGCTGCTTTTATGGCCCTGCTTCACAGGGACGAAGAGTGGAG
CACAGGCAAACCTCCTCAGTCTGGGAGGTCACTGCCCCAGGACCTGGACCTTTTAGAGAGCTCTCTC
GCCATCTTTTATCTCCCAGAGCTGCCATCTAACAATTGTCAAGGAACCTCATGTCTCACCTCAGGGG
CAGGGTACTCTCTCACTTAACCACCCTGGTCAAGTGAGCATCTTCTGGCTGGCTGTCTCCCCTCAC
ATGAAAACCCCAAACTTCTACCAATAACGGGATTTGGGTTCTGTTATGATAACTGTGACACACACA
ACACTCACACTCTGATAAAAGAGAACTCTGATAAAAGAGATGGAAGACACTAAAAAAAAAAAAAAA
AA
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ID NOS:1 & 3)

FIG. 2

GAGCCCCCTG CTCCGTCGCC TGCTGCTTGT TGCACTGCTG CAGCTGGCTC
GCACCCAGGC CCCTGTGTCC CAGTTTGATG GCCCCAGCCA CCAGAAGAAA
GTGGTGCCAT GGATAGACGT TTATGCACGT GCCACATGCC AGCCCAGGGA
GGTGGTGGTG CCTCTGAGCA TGGAACTCAT GGGCAATGTG GTCAAACAAC
TAGTGCCCAG CTGTGTGACT GTGCAGCGCT GTGGTGGCTG CTGCCCTGAC
GATGGCCTGG AATGTGTGCC CACTGGGCAA CACCAAGTCC GAATGCAGAT
CCTCATGATC CAGTACCCGA GCAGTCAGCT GGGGGAGATG TCCCTGGAAG
AACACAGCCA ATGTGAATGC AGACCAAAAA AAAAGGAGAG TGCTGTGAAG
CCAGACAGCC CCAGGATCCT CTGCCCGCCT TGCACCCAGC GCCGTCAACG
CCCTGACCCC CGGACCTGCC GCTGCCGCTG CAGACGCCGC CGCTTCCTCC
ATTGCCAAGG GCGGGGCTTA GAGCTCAACC CAGACACCTG TAGGTGCCGG
AAGCCGCGAA AGTGA
(SEQ ID NO:4)

FIG. 3

MSPLLRRLLL VALLQLARTQ APVSQFDGPS HQKKVVPWID VYARATCQPR
EVVVPLSMEL MGNVVKQLVP SCVTVQRCGG CCPDDGLECV PTGQHQVRMQ
ILMIQYPSSQ LGEMSLEEHS QCECRPKKKE SAVKPDSPRI LCPPCTQRRQ
RPDPRTCRCR CRRRRFLHCQ GRGLELNPDT CRCRKPRK
(SEQ ID NO:5)

FIG. 4

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ACCATGAGCC CCCTGCTCCG TCGCCTGCTG CTTGTTGCAC TGCTGCAGCT
GGCTCGCACC CAGGCCCCTG TGTCCCAGTT TGATGGCCCC AGCCACCAGA
AGAAAGTGGT GCCATGGATA GACGTTTATG CACGTGCCAC ATGCCAGCCC
AGGGAGGTGG TGGTGCCTCT GAGCATGGAA CTCATGGGCA ATGTGGTCAA
ACAACTAGTG CCCAGCTGTG TGACTGTGCA GCGCTGTGGT GGCTGCTGCC
TGACGATGG CCTGGAATGT GTGCCCACTG GGCAACACCA AGTCCGAATG
AGGTACCAG GGCCTATGGG TCAGATCCTC ATGATCCAGT ACCCGAGCAG
CAGCTGGGG GAGATGTCCC TGGAAGAACA CAGCCAATGT GAATGCAGAC
AAAAAAAAA GGAGAGTGCT GTGAAGCCAG ACAGCCCCAG GATCCTCTGC
CGCCTTGCA CCCAGCGCCG TCAACGCCCT GACCCCCGGA CCTGCCGCTG
CGCTGCAGA CGCCGCCGCT TCCTCCATTG CCAAGGGCGG GGCTTAGAGC
CAACCCAGA CACCTGTAGG TGCCGGAAGC CGCGAAAGTG A
SEQ ID NO:6)

FIG. 5

PLLRRLLL VALLQLARTQ APVSQFDGPS HQKKVVPWID VYARATCQPR
VVPLSMEL MGNVVKQLVP SCVTVQRCGG CCPDDGLECV PTGQHQVRMQ
GPMGQILM IQYPSSQLGE MSLEEHSQCE CRPKKKESAV KPDSPRILCP
TQRRQRPD PRTCRCRCRR RRFLHCQGRG LELNPDTCRC RKPRK
EQ ID NO:7)

FIG. 6

ACCATGAGCC CCCTGCTCCG TCGCCTGCTG CTTGTTGCAC TGCTGCAGCT
GGCTCGCACC CAGGCCCCTG TGTCCCAGTT TGATGGCCCC AGCCACCAGA
AGAAAGTGGT GCCATGGATA GACGTTTATG CACGTGCCAC ATGCCAGCCC
AGGGAGGTGG TGGTGCCTCT GAGCATGGAA CTCATGGGCA ATGTGGTCAA
ACAACTAGTG CCCAGCTGTG TGACTGTGCA GCGCTGTGGT GGCTGCTGCC
CTGACGATGG CCTGGAATGT GTGCCCACTG GGCAACACCA AGTCCGAATG
CAGATCCTCA TGATCCAGTA CCCGAGCAGT CAGCTGGGGG AGATGTCCCT
GGAAGAACAC AGCCAATGTG AATGCAGACC AAAAAAAAAA AGGAGAGTGC
TGTGA
(SEQ ID NO:8)

FIG. 7

SUBSTITUTE SHEET (RULE 26)

MSPLLRRLLL VALLQLARTQ APVSQFDGPS HQKKVVPWID VYARATCQPR
EVVVPLSMEL MGNVVKQLVP SCVTVQRCGG CCPDDGLECV PTGQHQVRMQ
ILMIQYPSSQ LGEMSLEEHS QCECRPKKKR RVL
(SEQ ID NO:9)

FIG. 8

```
mVEGF-B 167   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
mVEGF 164     - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
hPlGF         - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
mPDGF A       - M R T W A C L L L L G C G Y L A H A L A E E A E I P R E L
mPDGF B       M N R C W A L F L P L C C Y L R L V S A E G D P I P E E L Y

mVEGF-B 167   - - - - - - - - - - - - - - - - - - - - - - - - - - M S P L L R R L L
mVEGF 164     - - - - - - - - - - - - - - - - - - - - M N F L L S W V H W T L A L
mPlGF         - - - - - - - - - - - - - - - - - - - - M P V M R L F P C F L Q L
mPDGF A       I E R L A R S Q I H S I R D L Q R L L E I D S V G A E D A L
mPDGF B       E M L S D H S I R S F D D L Q R L L H R D S V D E D G A E L

mVEGF-B 167   L V A L L Q L A R T Q A P V S Q F D G P S H Q K K V V P W I
mVEGF 164     L L Y L H H A K W S Q A A P - T T E G E Q K S H E V I K F M
mPlGF         L A G L A L P A V P P Q Q W A L S - A G N G S S E V E V V P
mPDGF A       E T S L R A H G S H A I N H V P E K R P V P I R R K R S I E
mPDGF B       D L N M T R A H S G V E L E S S S R G R R S L G S L A A A E

mVEGF-B 167   D V Y A R A T - - C Q P R E V V V P L S M E L M G N V V K Q
mVEGF 164     D V Y Q R S Y - - C R P I E T L V D I F Q E Y P D E I E Y I
mPlGF         F Q E V W G R S Y C R A L E R L V D V V S E Y P S E V E H M
mPDGF A       E A I P A V - - - C K T R T V I Y E I P R S Q V D P T S A N
mPDGF B       P A V I A E - - - C K T R T E V F Q I S R N L I D R T N A N
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FIG. 9A

SUBSTITUTE SHEET (RULE 26)

```
mVEGF-B 167   L - - V P S C V T V Q R C G G C C P D D G L E C V P T G Q H
mVEGF 164     E - - K P S C V P L M R C A G C C N D E A L E C V P T S E S
mPlGF         F - - S P S C V S L L R C T G C C G D E N L H C V P V E T A
mPDGF A       F L I W P P C V E V K R C T G C C N T S S V K C Q P S R V H
mPDGF B       F L V W P P C V E V Q R C S G C C N N R N V Q C R A S Q V Q

mVEGF-B 167   Q V R M Q I L M I Q Y P S S Q - - - - L G E M S L E E H S Q
mVEGF 164     N I T M Q I M R I K P H Q S Q - - - H I G E M S F L Q H S R
mPlGF         N V T M Q L L K I R S G D R P - - - S Y V E L T F S Q H V R
mPDGF A       H R S V K V A K V E Y V R K K P K L K E V Q V R L E E H L E
mPDGF B       M R P V Q V R K I E I V R K K P I F K K A T V T L E D H L A

mVEGF-B 167   C E C R P K K K E S A V K P D S P R I L C P P C T Q R R Q R
mVEGF 164     C E C R P K K D R T - - K P E N H - - - C E P C S E R R K H
mPlGF         C E C R P L R E K M - - K P E - - - - R C G D A V P R R
mPDGF A       C A C A T S N L N P D H R E E E T D V R
mPDGF B       C K C E T I V T P R P V T R S P G T S R E Q R A K T P Q A R

mVEGF-B 167   - - - P D P R T C R C R C R R R R F L H C Q G R G L E L N P
mVEGF 164     L F V Q D P Q T C K C S C K N T D - S R C K A R Q L E L N E
mPlGF
mPDGF A
mPDGF B       V T I R T V R I R R P P K G K H R K F K H T H D K A A L K E

mVEGF-B 167   D T C R C R K P R K
mVEGF 164     R T C R C D K P R R
mPlGF
mPDGF A
mPDGF B       T L G A
```

FIG. 9B

ACCATGAGCC CTCTGCTCCG CCGCCTGCTG CTCGCCGCAC TCCTGCAGCT
GGCCCCCGCC CAGGCCCCTG TCTCCCAGCC TGATGCCCCT GGCCACCAGA
GGAAAGTGGT GTCATGGATA GATGTGTATA CTCGCGCTAC CTGCCAGCCC
CGGGAGGTGG TGGTGCCCTT GACTGTGGAG CTCATGGGCA CCGTGGCCAA
ACAGCTGGTG CCCAGCTGCG TGACTGTGCA GCGCTGTGGT GGCTGCTGCC
CTGACGATGG CCTGGAGTGT GTGCCCACTG GGCAGCACCA AGTCCGGATG
CAGATCCTCA TGATCCGGTA CCCGAGCAGT CAGCTGGGGG AGATGTCCCT
GGAAGAACAC AGCCAGTGTG AATGCAGACC TAAAAAAAAG GACAGTGCTG
TGAAGCCAGA CAGCCCCAGG CCCCTCTGCC CACGCTGCAC CCAGCACCAC
CAGCGCCCTG ACCCCCGGAC CTGCCGCTGC CGCTGCCGAC GCCGCAGCTT
CCTCCGTTGC CAAGGGCGGG GCTTAGAGCT CAACCCAGAC ACCTGCAGGT
GCCGGAAGCT GCGAAGGTGA
(SEQ ID NO:10)

FIG. 10

MSPLLRRLLL AALLQLAPAQ APVSQPDAPG HQRKVVSWID VYTRATCQPR
EVVVPLTVEL MGTVAKQLVP SCVTVQRCGG CCPDDGLECV PTGQHQVRMQ
ILMIRYPSSQ LGEMSLEEHS QCECRPKKKD SAVKPDSPRP LCPRCTQHHQ
RPDPRTCRCR CRRRSFLRCQ GRGLELNPDT CRCRKLRR
(SEQ ID NO:11)

FIG. 11

ATGAGCCCCC TGCTCCGTCG CCTGCTGCTT GTTGCACTGC TGCAGCTGGC
TCGCACCCAG GCCCCTGTGT CCCAGTTTGA TGGCCCCAGC CACCAGAAGA
AAGTGGTGCC ATGGATAGAC GTTTATGCAC GTGCCACATG CCAGCCCAGG
GAGGTGGTGG TGCCTCTGAG CATGGAACTC ATGGGCAATG TGGTCAAACA
ACTAGTGCCC AGCTGTGTGA CTGTGCAGCG CTGTGGTGGC TGCTGCCCTG
ACGATGGCCT GGAATGTGTG CCCACTGGGC AACACCAAGT CCGAATGCAG
ATCCTCATGA TCCAGTACCC GAGCAGTCAG CTGGGGGAGA TGTCCCTGGA
AGAACACAGC CAATGTGAAT GCAGACCAAA AAAAAAGGAG AGTGCTGTGA
AGCCAGACAG GGTTGCCATA CCCCACCACC GTCCCCAGCC CCGCTCTGTT
CCGGGCTGGG ACTCTACCCC GGGAGCATCC TCCCCAGCTG ACATCATCCA
TCCCACTCCA GCCCCAGGAT CCTCTGCCCG CCTTGCACCC AGCGCCGTCA
ACGCCCTGAC CCCCGGACCT GCCGCTGCCG CTGCAGACGC CGCCGCTTCC
TCCATTGCCA AGGGCGGGGC TTAG
(SEQ ID NO:12)

FIG. 12

ISPLLRRLLL VALLQLARTQ APVSQFDGPS HQKKVVPWID VYARATCQPR
VVVPLSMEL MGNVVKQLVP SCVTVQRCGG CCPDDGLECV PTGQHQVRMQ
LMIQYPSSQ LGEMSLEEHS QCECRPKKKE SAVKPDRVAI PHHRPQPRSV
GWDSTPGAS SPADIIHPTP APGSSARLAP SAVNALTPGP AAAAADAAAS
IAKGGA
SEQ ID NO:13)

FIG. 13

SUBSTITUTE SHEET (RULE 26)

```
ATGAGCCCTC TGCTCCGCCG CCTGCTGCTC GCCGCACTCC TGCAGCTGGC
CCCCGCCCAG GCCCCTGTCT CCCAGCCTGA TGCCCCTGGC CACCAGAGGA
AAGTGGTGTC ATGGATAGAT GTGTATACTC GCGCTACCTG CCAGCCCCGG
GAGGTGGTGG TGCCCTTGAC TGTGGAGCTC ATGGGCACCG TGGCCAAACA
GCTGGTGCCC AGCTGCGTGA CTGTGCAGCG CTGTGGTGGC TGCTGCCCTG
ACGATGGCCT GGAGTGTGTG CCCACTGGGC AGCACCAAGT CCGGATGCAG
ATCCTCATGA TCCGGTACCC GAGCAGTCAG CTGGGGGAGA TGTCCCTGGA
AGAACACAGC CAGTGTGAAT GCAGACCTAA AAAAAAGGAC AGTGCTGTGA
AGCCAGACAG GGCTGCCACT CCCCACCACC GTCCCCAGCC CCGTTCTGTT
CCGGGCTGGG ACTCTGCCCC CGGAGCACCC TCCCCAGCTG ACATCACCCA
CCCACTCCA GCCCCAGGCC CCTCTGCCCA CGCTGCACCC AGCACCACCA
CGCCCTGAC CCCCGGACCT GCCGCCGCCG CTGCCGACGC CGCAGCTTCC
CCGTTGCCA AGGGCGGGGC TTAG
SEQ ID NO:14)
```

FIG. 14

```
SPLLRRLLL AALLQLAPAQ APVSQPDAPG HQRKVVSWID VYTRATCQPR
VVVPLTVEL MGTVAKQLVP SCVTVQRCGG CCPDDGLECV PTGQHQVRMQ
LMIRYPSSQ LGEMSLEEHS QCECRPKKKD SAVKPDRAAT PHHRPQPRSV
GWDSAPGAP SPADITHPTP APGPSAHAAP STTSALTPGP AAAAADAAAS
VAKGGA
SEQ ID NO:15)
```

FIG. 15

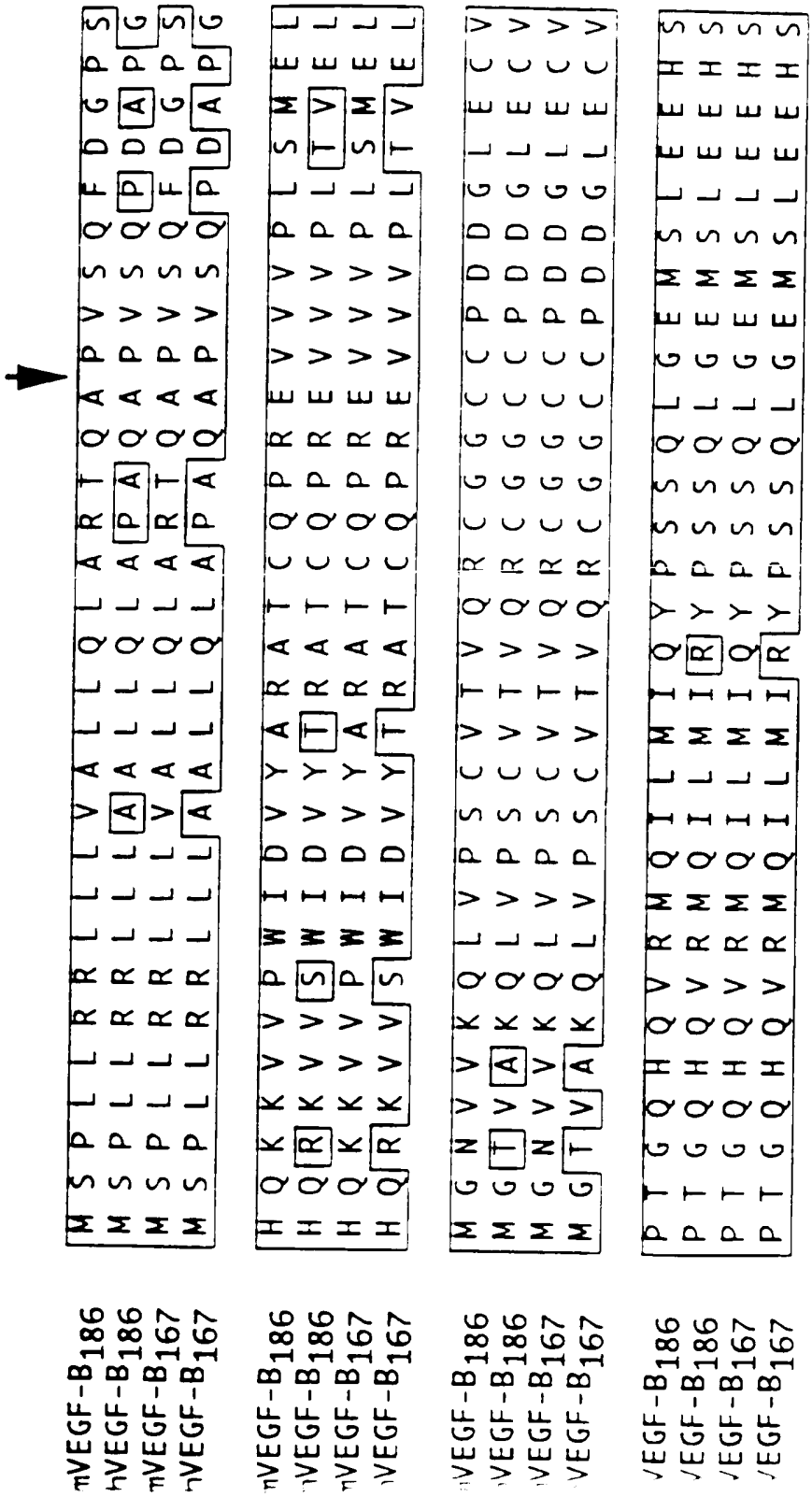
MSPLLRRLLLVALLQLARTQAPVSQFDGPS
MSPLLRRLLLALLQLAPAQAPVSQPDAPG
MSPLLRRLLLVALLQLARTQAPVSQFDGPS
MSPLLRRLLLALLQLAPAQAPVSQPDAPG
HQKKVVPWIDVYARATCQPREVVVPLSMEL
HQRKVVSWIDVYTRATCQPREVVVPLTVEL
HQKKVVPWIDVYARATCQPREVVVPLSMEL
HQRKVVSWIDVYTRATCQPREVVVPLTVEL
MGNVVKQLVPSCVTVQRCGGCCPDDGLECV
MGTVAKQLVPSCVTVQRCGGCCPDDGLECV
MGNVVKQLVPSCVTVQRCGGCCPDDGLECV
MGTVAKQLVPSCVTVQRCGGCCPDDGLECV
PTGQHQVRMQILMIQYPSSQLGEMSLEEHS
PTGQHQVRMQILMIRYPSSQLGEMSLEEHS
PTGQHQVRMQILMIQYPSSQLGEMSLEEHS
PTGQHQVRMQILMIRYPSSQLGEMSLEEHS
hVEGF-B186
hVEGF-B186
hVEGF-B167
hVEGF-B167
hVEGF-B186
hVEGF-B186
hVEGF-B167
hVEGF-B167
hVEGF-B186
hVEGF-B186
hVEGF-B167
hVEGF-B167
hVEGF-B186
hVEGF-B186
hVEGF-B167
hVEGF-B167

FIG. 16A

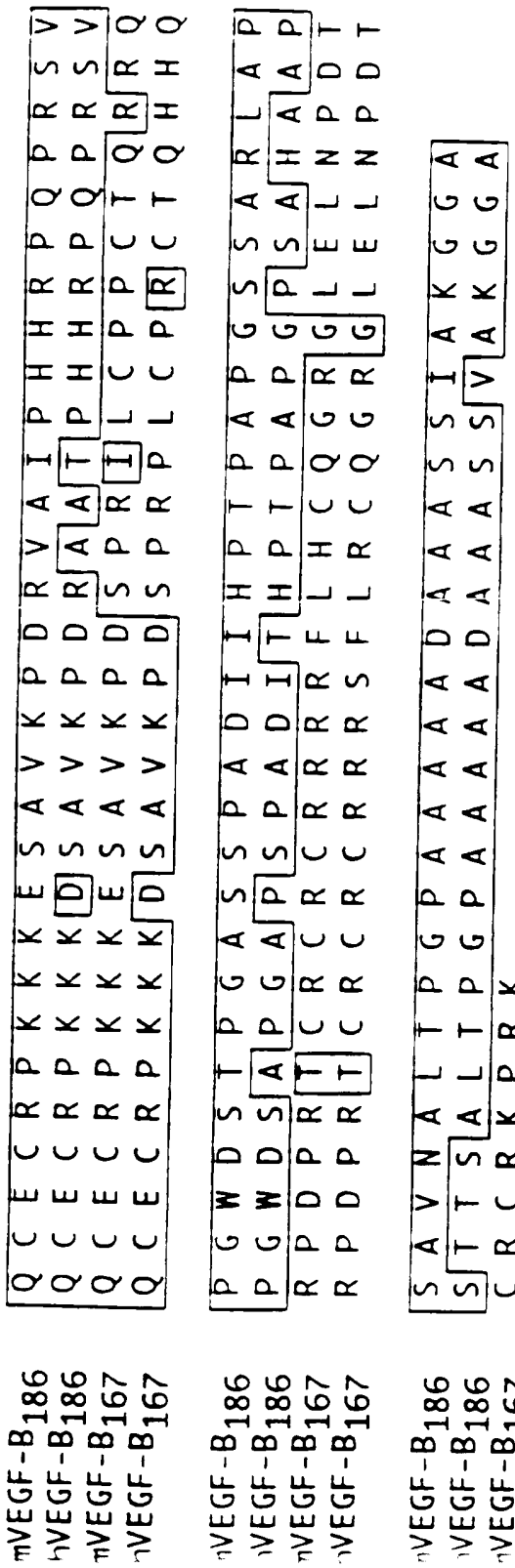
mVEGF-B186
hVEGF-B186
mVEGF-B167
hVEGF-B167
Q C E C R P K K K E S A V K P D R V A I P H H R P Q P R S V
Q C E C R P K K K K D S A V K P D R A A T P H H R P Q P R S V
Q C E C R P K K K K E S A V K P D S P R I L C P P C T Q R R Q
Q C E C R P K K K K D S A V K P D S P R P L C P R C T Q H H Q
mVEGF-B186
hVEGF-B186
mVEGF-B167
hVEGF-B167
P G W D S T P G A S S P A D I I H P T P A P G S S A R L A P
P G W D S A P G A P S P A D I T H P T P A P G P S A H A A P
R P D P R T C R C R C R R R F L H C Q G R G L E L N P D T
R P D P R T C R C R C R R R S F L R C Q G R G L E L N P D T
hVEGF-B186
hVEGF-B186
mVEGF-B167
hVEGF-B167
S A V N A L T P G P A A A A A D A A A S S I A K G G A
S T T S A L T P G P A A A A A D A A A S S V A K G G A
C R C R K P R K
C R C R K L R R

FIG. 16B

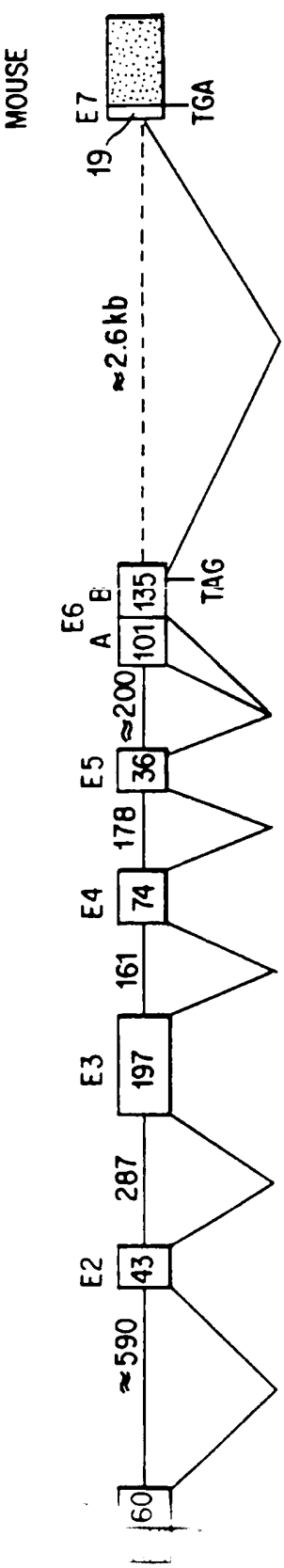

FIG.17

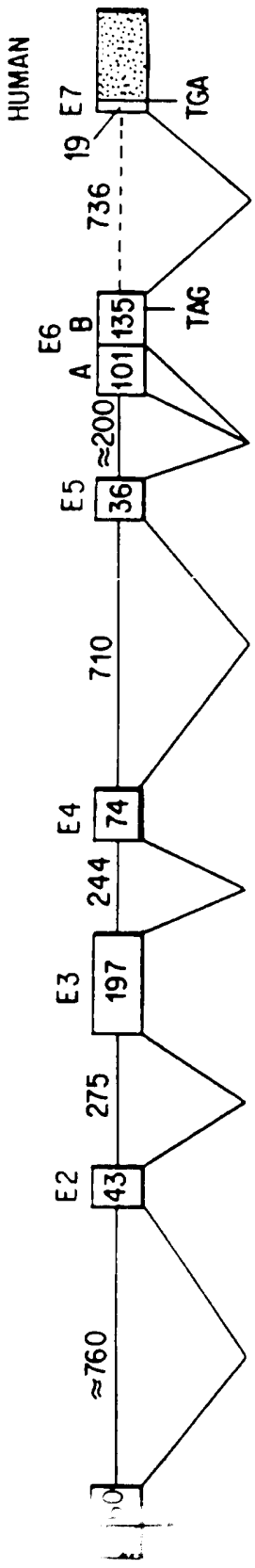

FIG. 17A

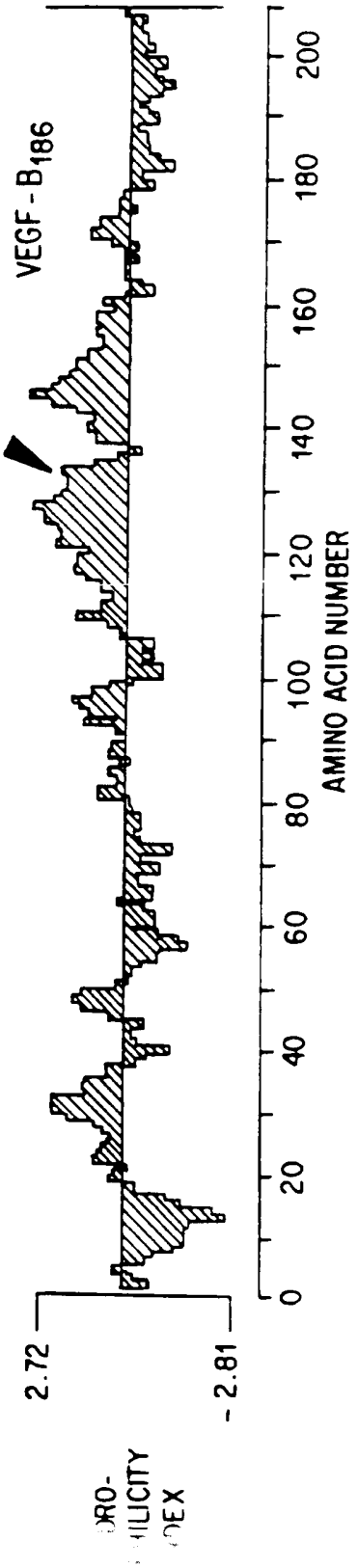

FIG. 18

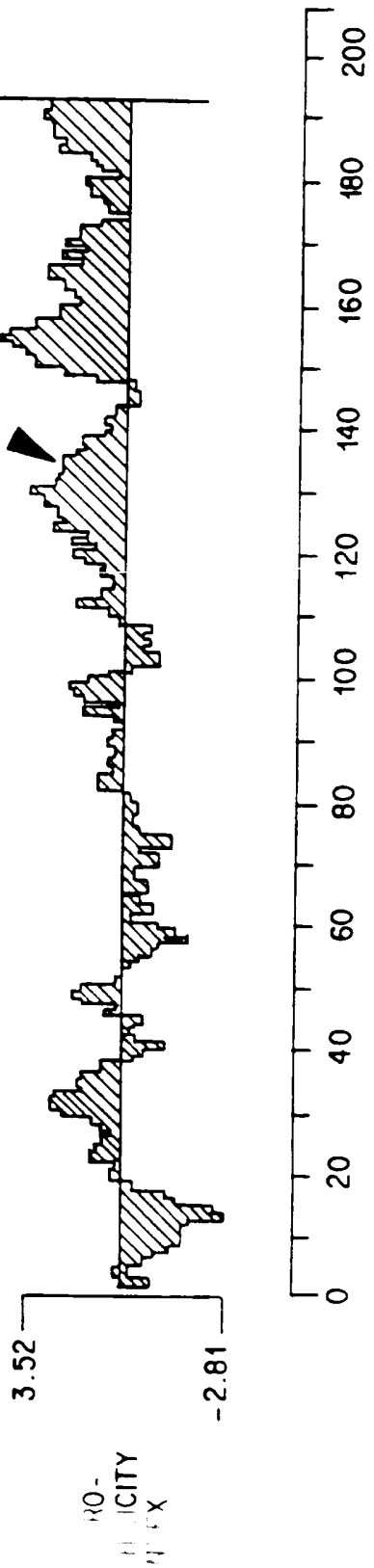

FIG. 18A

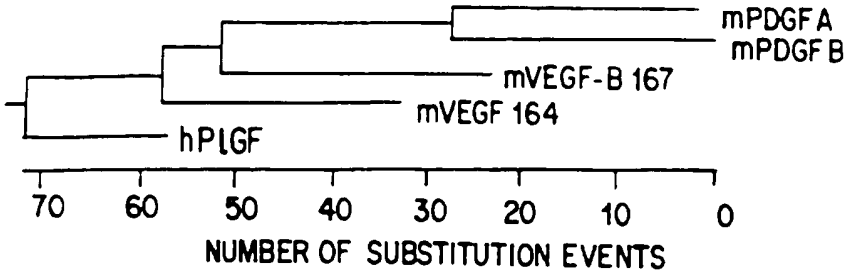
mPDGF A
mPDGF B
mVEGF-B 167
mVEGF 164
hPLGF
70
60
50
40
30
20
10
0
NUMBER OF SUBSTITUTION EVENTS

FIG. 19

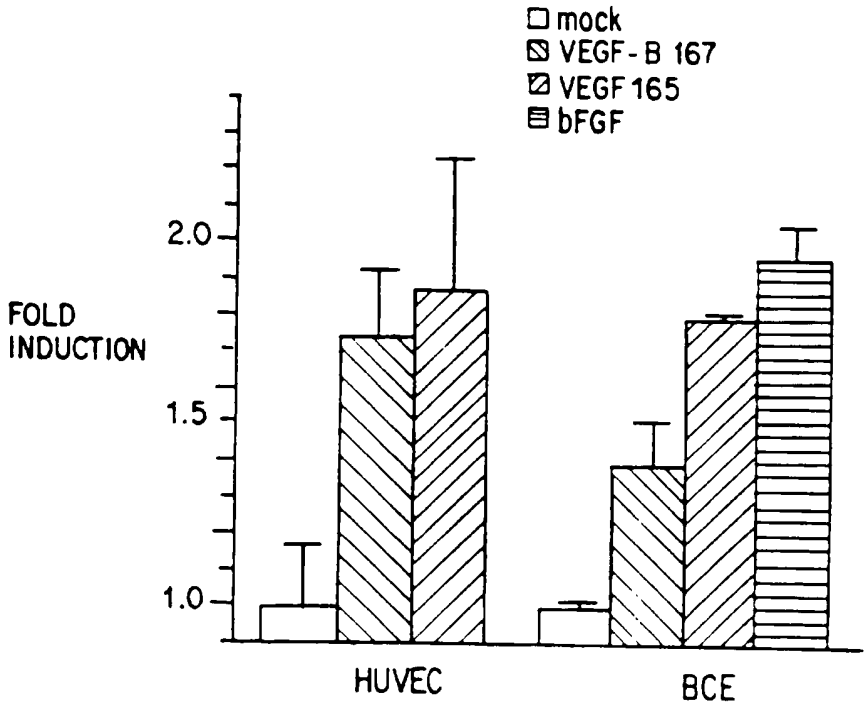
mock
VEGF-B 167
VEGF 165
bFGF
FOLD INDUCTION
2.0
1.5
1.0
HUVEC
BCE

FIG. 20

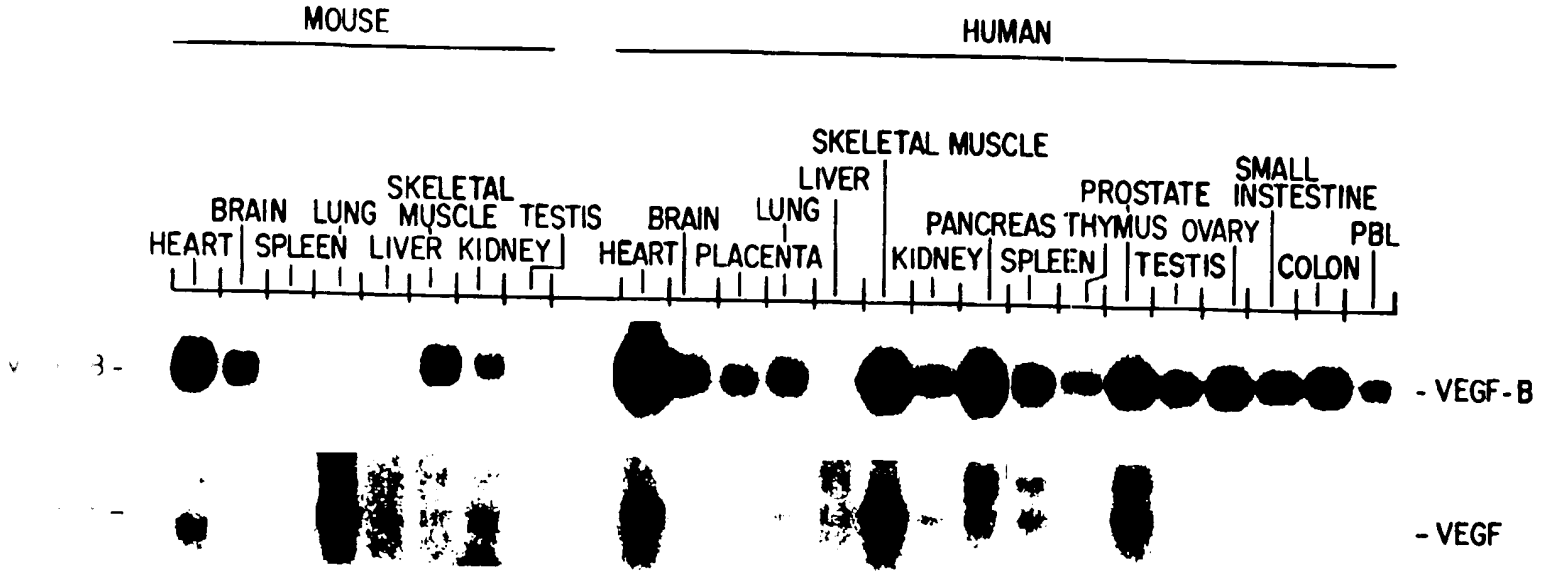

FIG. 21

SUBSTITUTE SHEET (RULE 26)

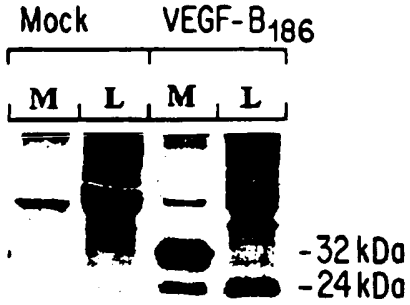

FIG. 22

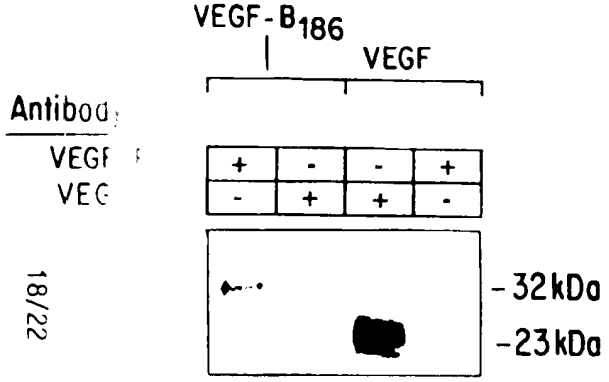

FIG. 23A

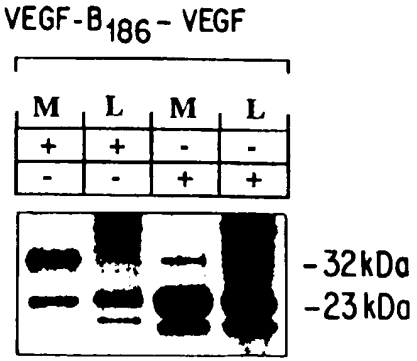

FIG. 23B

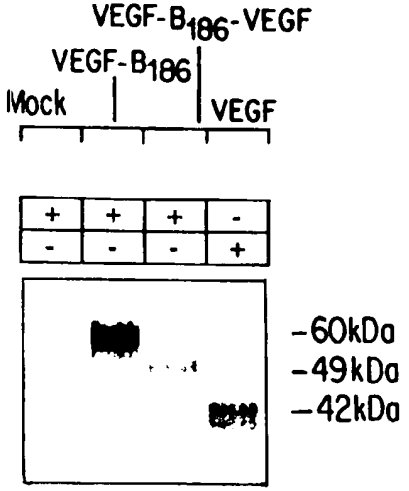

FIG. 23C

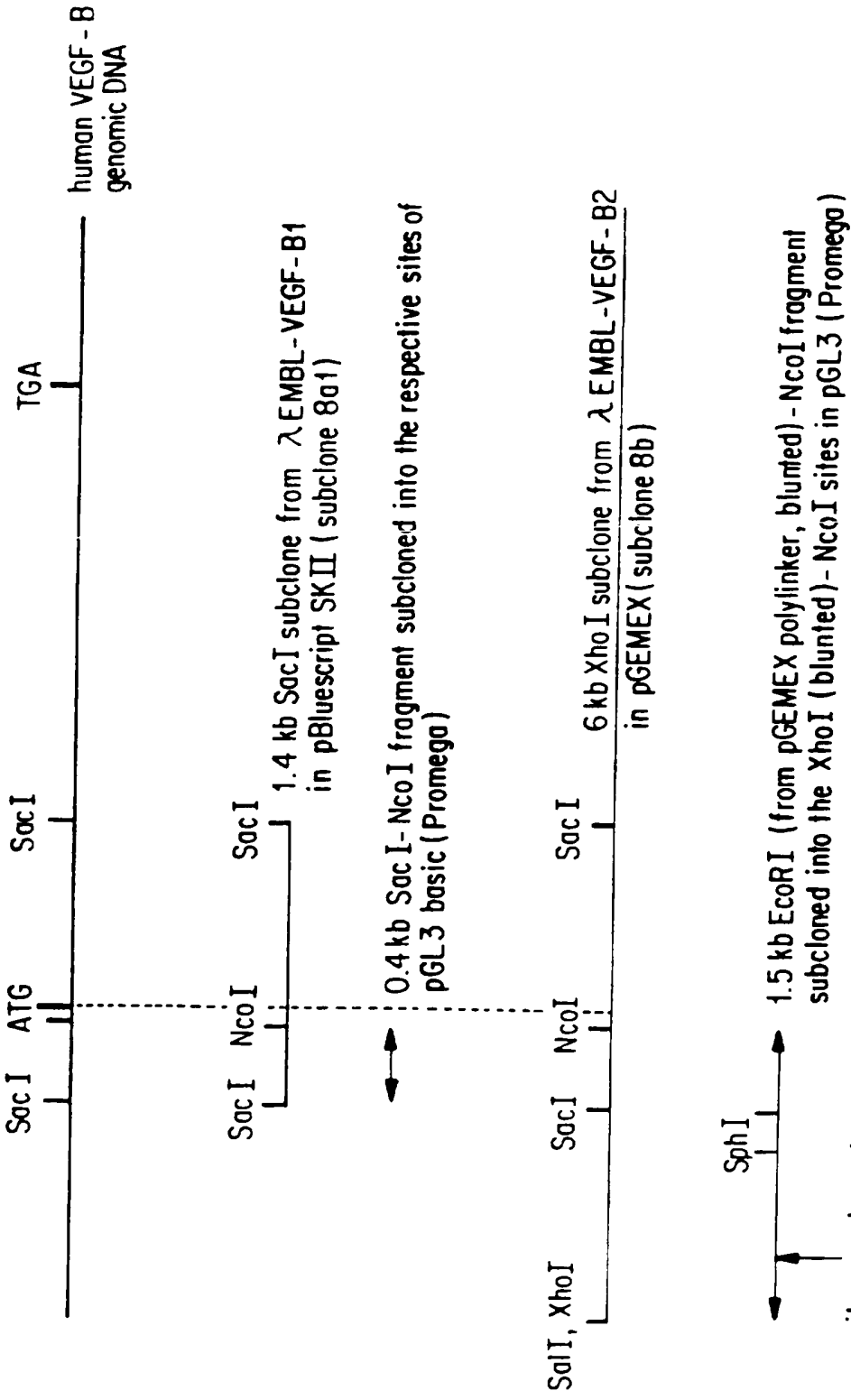
human VEGF-B genomic DNA
TGA
Sac I
ATG
Sac I
Nco I
Sac I
1.4 kb Sac I subclone from λ EMBL-VEGF-B1 in pBluescript SK II (subclone 8a1)
0.4 kb Sac I-Nco I fragment subcloned into the respective sites of pGL3 basic (Promega)
Sac I
Nco I
Sac I
6 kb Xho I subclone from λ EMBL-VEGF-B2 in pGEMEX (subclone 8b)
Sal I, Xho I
Sph I
1.5 kb EcoR I (from pGEMEX polylinker, blunted) - Nco I fragment subcloned into the Xho I (blunted) - Nco I sites in pGL3 (Promega)
silencer element

FIG. 24

ctcgagatct gtttgttgtc ttggaacaat acggtttaga ggtgactggc

Xho I

gggtgacgag aacatatgcg agttcaccta agagaaaagc tgaatgaggc

aatgcctctt cctgaccata tctcttactc agataactat agaatttatt

gtccagtaaa gggtatatta aaaaatcata ttaaaagtca tacagtgaag

silencer binding site 2

ttgtccaggg aaatcaagac ttaacagtct cactctgaca ataatgaaca

gggggattcc ctcaagatag actaggacat gaccccacac tggcaggtag

tagtaccaga aaagaacgca tggaaaatct ttaccttatg cttgaggtag

ggaccaggct aaagtgaagg ccagacctaa aattctatct aaaataaatc

cacaatcgaa gaaaatatgt ggtgtacagg tatagaatgt ctttactgga

tcattgaaat agtaagataa attcaacttt ttacattgtt ttcttttcct

ccagttaggg cttgagacct tcgtctctgg agagtgactg tcaattggag

ccctgctttc tgggtttctg gccaggggggg ttgtggatgc ttaacatgtg

cctttcacag gacacttcct taccccagca gtggccangt gtgcatccca

FIG.25A

651 cgaccaggcc tccctctcac ggaacatctg ttgagactag gagatgcctg

701 gtgactgttg cctgacctgt gtcctgtgta tttctgacaa gagccactct

751 caaagaccct ggccaggagg agagttaggt tccagtgtag gtcagctcag

801 acagatggag gccacagaan caaacatggg aaatcacaga agtaggttta

851 ttactcacag atccctatcc caaccaccca ggtgccctct cctccagggc

901 caacagaggc atccttcagc aggagcgaca acggctaggg cagcggcaag

951 ccgccaccat ccgagccaac ccaggccccg agatcgtgcc ccggggcgcc

1001 ggcccctgag gggctcacct ggatggggcc tgcatgcgtt cccgctttgc

1051 ttccttccct ggacggcccg ctcccccgaa acgcgccgcc aataaagtga

1101 ttcgcagagc tcgtgtgcgg ctccctcctt aaggcccgac gccccggcc

1151 ccggcctcgc caagggcagc gccccggcct ccgggtagtg gcggccggcg

1201 actggggagc ccagcctcct gggcggtgcg tcccccttccc cctgccgcgg

1251 cgggaggcgg gaggggggtgt gtggaggagg cgggcccccgc cgacggcctc

SacI

Sph I

FIG. 25B

```
1301  gccccccccac cccgccgcccc cgccccccgcc ccacgggccc ggtggggagc
1351  gcgtgtctctgg gtcacatgag ccgcctgccc gccagccccgg gcccagcccc
1401  ccgccgcccc cgccgtcccc gccgccgctg cccgccgcca ccggccgccc
1451  gcccgccccgg ctcctccggc cgccttcgct gcgctgcntg cgctgcctgc
1501  acccagggct cgggaggggg ccgcgcggaga gccgccccccc gcgccccggcc
...   Nco I site is about 30 bp downstream)   (SEQ ID NO:17)
```

FIG. 25C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/02957

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 7.1, 240.2, 320.1; 514/2; 530/350, 387.9, 388.1, 388.24; 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FERRARA et al. The Vascular Endothelial Growth Factor Family of Polypeptides. Journal of Cellular Biochemistry. 1991, Vol. 47, pages 211-218, especially the abstract.	1-57
A, P	WO 95/24473 A (HUMAN GENOME SCIENCES, INC.) 14 September 1995, see entire document.	1-57
A	KLAGSBRUN et al. Regulators of Angiogenesis. Annual Review of Physiology. 1991, Vol. 53, pages 217 to 239, see entire document.	1-57
A	FOLKMAN et al. Angiogenesis. The Journal of Biological Chemistry. 05 June 1992, Vol. 267, No. 16, pages 10931 to 10934, see entire document.	1-57

☐ Further documents are listed in the continuation of Box C ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search: 04 JUN 1996

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
TERRY A. MCKELVEY
Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/02957

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.

Remark on Protest

[illegible] protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/02957

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 38/17; C07K 14/47, 16/18; C12N 5/02, 5/06, 15/12, 15/70, 15/79; C12Q 1/68; G01N 33/53

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/6, 7.1, 240.2, 320.1; 514/2; 530/350, 387.9, 388.1, 388.24; 536/23.5

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, DIALOG
search terms: VEGF, VEGF-B, vector?, plasmid?, pharmaceutic?, antibod?, monoclonal?, 293EBNA, insect, COS endothelial, mesodermal, diagnost?, detect?, polymerase chain reaction, dimer?, heparin

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-17, 35-40 and 43, drawn to DNA encoding VEGF-B protein, vector comprising said DNA, host cell comprising said protein, diagnostic method using said DNA.

Group II, claims 18-32 and 45-53, drawn to VEGF-B protein and method of promoting release of said protein.

Group III, claims 33-34, 41-42 and 44, drawn to anti-VEGF-B antibody, diagnostic method using said antibody.

Group IV, claims 54-56, drawn to antisense vector and method of retarding expression using said vector.

Group V, claim 57, drawn to DNA comprising VEGF-B promoter fragment.

The inventions listed as Groups I-V do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the DNA encoding VEGF-B, VEGF-B protein, antibody against VEGF-B, vector encoding VEGF-B antisense molecule, and DNA comprising VEGF-B promoter are chemically, biochemically, structurally, and functionally different and distinct from each other and thus share no common technical feature. The methods of each Group also do not share a common technical feature because each method comprises steps different from the steps of the other methods, and the products of the other groups are neither required for nor used in the methods of the inventions of the other groups. Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.